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<b>(21) International Application Number:</b> PCT/US93/01925 <b>(22) International Filing Date:</b> 8 March 1993 (08.03.93)  <b>(30) Priority data:</b> 847,311 6 March 1992 (06.03.92) US  <b>(71) Applicant:</b> THE GOVERNMENT OF THE UNITED STATES OF AMERICA as represented by THE DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; National Institutes of Health, Box OTT, Bethesda, MD 20892 (US).  <b>(72) Inventors:</b> BERZOFSKY, Jay ; 9321 Corsica Drive, Bethesda, MD 20814 (US). TAKESHITA, Toshiyuki ; 263 Congressional Lane, Rockville, MD 20852 (US). SHIRAI, Mutsunori ; 381 Congressional Lane, Rockville, MD 2052 (US). PENDLETON, C., David ; 9100 Old Georgetown Road, Bethesda, MD 20814 (US). MARGULIES, David, H. ; 7805 Radnor Road, Bethesda, MD 20817 (US). KOZLOWSKI, Steven ; 2501 Q Street, N.W., #105, Washington, DC 20007 (US).			<b>(74) Agents:</b> SVENSSON, Leonard, R. et al.; Birch, Stewart, Kolasch & Birch, P.O. Box 747, Falls Church, VA 22046 (US).  <b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> POTENT PEPTIDE FOR STIMULATION OF CYTOTOXIC T LYMPHOCYTES SPECIFIC FOR THE HIV-1 ENVELOPE  <b>(57) Abstract</b>  Peptides having high activity in the eliciting of a cytotoxic T lymphocyte response to the HIV-1 envelope glycoprotein gp160 are described. The activation of 12-15 residue peptides by proteolytic degradation to shorter peptides is shown as are general techniques for characterizing such activation processes.			

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POTENT PEPTIDE FOR STIMULATION OF CYTOTOXIC T  
LYMPHOCYTES SPECIFIC FOR THE HIV-1 ENVELOPE

RELATED APPLICATIONS

This application is a Continuation-In-Part application of the U.S. patent application bearing Serial Number 07/760,530, filed on September 18, 1991, which is in turn a Continuation-In-Part of U.S. patent application 07/148,692, filed on January 26, 1988. These applications are incorporated in their entirety herein by reference.

BACKGROUND OF THE INVENTION

10 Field of the Invention

The invention relates to peptides useful as vaccines for the prophylaxis and/or treatment of Human Immunodeficiency Virus infection in humans, to compositions incorporating such peptides and to methods for the administration of such vaccines.

15 Related Art

This application makes reference to various literature publications, which are herein incorporated in their entirety by reference.

20 Live virus vaccines and killed whole or subunit virus vaccines for AIDS have potential safety risks. In contrast, synthetic peptides are inherently safe. Furthermore, molecules

corresponding to whole viral proteins but made by recombinant DNA technology contain, in addition to protective epitopes, structures which potentially will elicit suppression of the immune response, or which will elicit antibodies that, rather than being protective, may enhance viral uptake and thus be deleterious. A vaccine which contains only selected peptides that elicit the appropriate type of immunity and do not have other deleterious effects should be more effective for a difficult virus such as HIV.

Our previous work showed that the major CTL antigenic determinant of HIV-1 envelope protein gp 160 consisted of residues 315-329 in the numbering sequence of Ratner et al. (27). However, we have now found that this peptide does not bind intact to the class I MHC molecule that must present it to CTL, but rather it must first be proteolytically cleaved, by proteases such as those present in serum.

T-cell stimulation by the HIV-1 gp160-derived p18 peptide presently by H-2D<sup>d</sup> class I major histocompatibility complex (MHC) molecules in a cell-free system was found to require proteolytic cleavage. This extracellular processing was mediated by peptidases present in fetal calf serum (FCS). In vitro processing of p18 resulted in a distinct reverse phase HPLC profile, from which a biologically active product was isolated and sequenced. This peptide processing can be specifically blocked by the angiotensin converting enzyme (ACE) inhibitor captopril and can occur by exposing p18 to purified ACE. The ability of naturally occurring extracellular proteases to convert inactive peptides to T-cell antigens has

important implications for understanding cytotoxic T-lymphocyte (CTL) responses in vivo and for rational peptide vaccine design.

Although naturally processed peptides  
5 associate with newly formed MHC class I molecules intracellularly (1), extracellular loading of surface class I molecules by synthetic peptides (2) is commonly used to analyze MHC class I peptide interactions. Recent data have provided  
10 substantial evidence that peptides bound to class I are approximately nine amino-acids in length (3-9), but larger peptides are capable of sensitizing targets for class I MHC-restricted lysis. In some cases the activity of these longer peptides can be  
15 traced to the presence of contaminating shorter products which are extremely biologically potent (9).

The HIV-1 (IIIB) gp160 envelope glycoprotein-derived peptide, p18, is 15 amino  
20 acids in length (residues 315-329). It is the immunodominant CTL determinant of gp160 in H-2D<sup>d</sup> mice (10,11) and can sensitize syngeneic cells for lysis by CTL from HIV-1-infected humans (12). Previous studies of the ability of this peptide to  
25 form stimulatory complexes with purified H-2D<sup>d</sup> molecules in vitro, indicated that two activities of FCS were required for recognition of p18 by a specific T-cell hybridoma. One activity was that of  $\beta$ 2-microglobulin ( $\beta$ 2-m) (13,14-17) and the other  
30 activity could be performed by ovalbumin. Most batches of bovine serum albumin (BSA) were unable to replace this  $\beta$ 2-m independent effect of FCS.

We have tested 9, 10 and 11 residue peptides, derived from p18, overlapping or contained within  
35 the p18-I-10 peptide, including specifically both

possible 9 residue peptides contained within p18-I-10, and all of these have been found to be less active than p18-I-10. This finding concerning the importance of length in the activity of peptides presented by MHC class I molecules and the identification of a truncation of p18, p18-1-10 (residues 318-327), with 10 to  $10^2$ -fold greater potency of T-cell stimulation prompted us to consider the possibility that ovalbumin and FCS were processing p18 to an active, shorter peptide.

Cytotoxic T lymphocytes (CTL) and T helper cells recognize processed antigenic peptides in association with the products of the major histocompatibility complex (MHC) (26-30). Generally, CD8+ CTL are restricted by MHC class I molecules, such as H-2K,-D,-L in mice and HLA-A,-B,-C in humans, presented on the surface of antigen-presenting cells (APC), while CD4+T helper cells (Th) are restricted by MHC class II molecules, such as I-A or I-E in mice and HLA-DR,-DQ or -DP in humans. T cells are able to recognize a wide variety of antigens in the context of relatively few MHC molecules by means of specific T cell receptors (TCR) (31-34). There is no known difference in overall TCR repertoire between CD4+ and CD8+ T cells.

Although it has generally been assumed that there is no reason to expect the same peptides to be presented by both class I and class II MHC molecules, there are a few cases reported in which peptides presented by class I molecules were found to be presented by or to bind to class II molecules also (35,36). Moreover, we have recently found that the immunodominant antigenic determinant of HIV-I envelope protein gp160 recognized by BALB/c



murine as well as human CD8+ CTL with class I MHC molecules (peptide P18IIIB, residues 315-329, RIQRGPGRAFVTIGK) (37,38), is also presented by class II MHC molecules of both mice (39) and humans (40) to CD4+ helper T cells. Conversely, we found that three other peptides of HIV-1 gp160 that were originally identified as stimulating CD4+ helper T cells of mice (41,42) and humans (40,43) also were presented by human class I molecules to human CD8+ CTL (38). Thus, we asked whether these latter peptides also were presented by murine class I molecules to CD8+ CTL, and if so, what range of class I molecules could present them.

These findings also led us to raise a related but distinct question. A few cases have been described of antigenic determinants that happen to be broadly or permissively presented by multiple class II MHC molecules, especially in the case of murine I-E or human DR, in which polymorphism is limited to the beta chain, but the alpha chain is conserved (44,45). However, no similar cases have been studied for presentation by class I MHC molecules, and no analysis of 10 different class I MHC haplotypes as here has previously been reported. Because both domains of the MHC peptide-binding site are polymorphic in class I molecules, exploring permissiveness in class I presentation would be of interest in comparison with class II. Also such widely presented antigenic determinants would clearly be useful for development of synthetic vaccines aimed at a broad outbred population of diverse MHC types. This is especially relevant for HIV-1, because whole virus and even whole envelope protein can elicit deleterious immune responses that can enhance

infection or contribute to the development of immune deficiency (reviewed in (46)).

Therefore, for both theoretical and potential practical interest, we explored the breadth of presentation by class I MHC molecules from ten distinct murine MHC haplotypes of both the original CTL determinant peptide P18, and two of the original helper T-cell determinant peptides T1 (428-443, KQIINMWQEVGKAMYA), and HP53 (HP53, 834-848, also known as TH4.1, DRVIEVVQGAYRAIR). P18 and HP53 were presented by at least 4 different class I MHC molecules in mice immunized with recombinant vaccinia virus transfected with HIV-1 gp 160, and T1 was recognized by CD8+ CTL in mice of three MHC haplotypes. Indeed, even the same segments of the peptides are recognized by the several haplotypes. Thus, permissiveness of presentation by class I molecules appears to be at least as great as that reported for presentation by class II molecules, and the extent of overlap between the repertoire of sites presented by class I and the repertoire of sites presented by class II may be much greater than suspected. Also, from a practical point of view, these peptides that are broadly presented by multiple class I as well as class II MHC molecules may be versatile components of a vaccine.

#### SUMMARY OF THE INVENTION

The invention is defined by the properties of peptides of the immunodominant epitopes of the Human Immunodeficiency Virus (HIV) 160 kilodalton envelope glycoprotein (gp160). The purpose of the invention is to develop a vaccine to prevent AIDS based partly or solely on synthetic or recombinant

peptides. Cytotoxic T lymphocytes (CTL) may be a primary means of host defense against HIV. The present invention provides the most potent peptide known to induce cytotoxic T cells specific for HIV-1 gp160 envelope protein, and that can kill cells expressing this envelope protein.

Accordingly, one object of the invention is to provide peptides which provide advantageous immune responses, eliciting cytotoxic T lymphocyte response at concentrations in the range of  $10^{-12}$  to  $10^{-6}$  M. Preferred embodiments of this aspect of the invention are the ten residue peptides which represent the highly immunogenic regions of the V3 loop of various HIV isolates; RGPGRFVTI (IIIb isolate), IGPGRFYTT (MN isolate), IGPGRFYAT (SC isolate), KGPRVIYAT (RF isolate), IGPGRFHTT (SF2 isolate), IGPGRTLYAR (NY5 isolate), LGPRVWYTT (CDC4 isolate), IGPGRFRTT (WMJ2 isolate).

A second object of the invention is to provide peptides which elicit an immune response characterized by activation of both class I-restricted T lymphocytes and class II-restricted T lymphocytes; class I-restricted T lymphocytes elicit a  $CD8^{+}$  cytotoxic T lymphocyte response, class II-restricted T lymphocytes elicit  $CD4^{+}$  T helper lymphocytes, which play a role in both the production of cytotoxic T lymphocytes and in the production of antibodies by B cells.

A third object of the invention is to provide peptides which are activated by cleavage of the peptide by a protease to produce a more active peptide. Such peptides comprise the residues 315-329 (numbered according to Ratner et al. (25)) of the HIV-1 gp160 envelope protein. Preferred embodiments of this aspect of the invention are

peptides RIQRGPGRAFTIGK (isolate IIIB),  
 RIHIGPGRIFYTTKN (isolate MN),  
 SITKGPGRVIYATGQ (isolate RF),  
 SIHIGPGRIFYATGD (isolate SC),  
 5 SLSIGPGRAFTREI (isolate WMJ-2),  
 SISIGPGRAFFATTD (isolate Z321),  
 SIYIGPGRAFHTTGR (isolate SF2),  
 GIAIGPGRTLYAREK (isolate NY5),  
 RVTLGPGRVWYTTGE (isolate CDC4),  
 10 SIRIGPGKVFTAKGG (isolate Z3),  
 GIHFGPGQALYTTGI (isolate MAL),  
 STPIGLGQALYTTRG (isolate Z6),  
 STPIGLGQALYTTRI (isolate JY1), and  
 RTPTGLGQSLYTTRS (isolate ELI) being activated by  
 15 angiotensin converting enzyme.

Further objects of the invention are to  
 provide compositions including such peptides and to  
 provide methods of treatment and/or prophylaxis of  
 HIV infection in humans which utilize such  
 20 peptides.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the growth inhibition response  
 of peptide p18 and truncations of that peptide in  
 BSA solution.

25 Figure 2 shows the growth inhibition response  
 to peptide p18 or peptide p18-I-10 following  
 treatment with FCS, BSA or ovalbumin. In a, B4.2.3  
 growth inhibition response to p18 is dependent on  
 ovalbumin or FCS. (---O---), p18 in 0.5% BSA; (-  
 30 □—), p18 in 0.5% ovalbumin; (—▲—), p18 in  
 0.5% FCS. In b, B4.2.3 growth inhibition response  
 to p18-I-10 is decreased by ovalbumin or FCS. (---  
 ●---), 18-1-10 in 0.5% BSA; (—■—), 18-1-10 in  
 0.5% ovalbumin; (—▲—), 18-1-10 in 0.5% FCS.

35 Figure 3 shows chromatograms of peptides

treated with BSA or FCS. In **a**, reverse phase HPLC fractions of ovalbumin treated p18 and their ability to functionally bind H-2D<sup>d</sup>. (\_\_\_\_), 220 nm absorbance; (—○—), 1:5 dilution of fractions in 0.5% BSA added to H-2D<sup>d</sup> coated plates; (—●—), 1:25 dilution of fractions in 0.5% BSA added to H-2D<sup>d</sup> coated plates. In **b**, reverse phase HPLC fractions of BSA treated p18 and their ability to functionally bind H-2D<sup>d</sup>. (\_\_\_\_), 220 nm absorbance; (—△—), 1:5 dilution of fractions in 0.5% BSA added to H-2D<sup>d</sup> coated plates; (—▲—), 1:25 dilution of fractions in 0.5% BSA added to H-2D<sup>d</sup> coated plates.

Figure 4 shows the effect of carboxypeptidase inhibitors on p18 functional binding to H-2<sup>d</sup> expressing cells. In **a**, effect of carboxypeptidase inhibitors in p18 functional binding to H-2D<sup>d</sup> on FCS. (—○—), potato carboxypeptidase inhibitor; (—●—), Plummer's inhibitor; (—△—), captopril (—▲—), E-64. In **b**, Angiotensin converting enzyme (ACE) processes p18 into an active form in BSA. (—○—), p18 + ACE; (—○—), p18-I-10 + ACE; (—△—), p18 + carboxypeptidase N; (—▲—), 18-1-10 + carboxypeptidase N.

Figure 5a. shows that B4.2.3 lymphokine response to p18 and H-2D<sup>d</sup> positive L-cells in FCS is decreased by captopril. (—○—), p18; (—□—), p18 + captopril; (—△—), p18 + Plummer's inhibitor; (—●—), p18-I-10; (---■---), p18-I-10 + captopril; (---▲---), p18-I-10 + Plummer's inhibitor. In 5b, The B4.2.3 lymphokine response to gp-160 transfected H-2D<sup>d</sup> positive 3T3 cells is not decreased by captopril. (—○—), gp-160 transfectant; (—□—), gp-160 transfectant +

captopril; (—△—), gp-160 transfectant + Plummer's inhibitor, (---O---), Neo transfectant. CTLL-2 thymidine incorporation in the absence of transfected L-cells was <500 c.p.m..

Figure 6 shows the activation of a murine cytomegalovirus responsive CTL by peptides derived from murine cytomegalovirus when incubated in the presence of FCS with and without captopril.

Figure 7 shows the sequences of the variant forms of the p18 peptide utilized in several experiments.

Figure 8 shows the recognition of the 10-residue core peptide p18-I-10 by cytotoxic T lymphocytes of four different class I MHC types. Effectors from each CTL line were added to <sup>51</sup>Cr-labelled 18Neo (Balb/c 3T3 fibroblasts) and lysis was assessed in the presence of the indicated concentrations of peptides at an effector to target ratio of 5:1.

Figure 9 shows the the interferon production by a HIV-1, strain MN-specific CTL line in response to presentation of the MN strain peptides homologous to p18 and p18-I-10.

Figure 10 shows the activation, measured by IL-2 production, of gp160-immune CD4<sup>+</sup> T cells stimulated by peptides p18 and p18-I-10.

#### DETAILED DESCRIPTION OF THE INVENTION

Proteolytic cleavage of peptides circulating in vivo is an inefficient process, and therefore therapeutic applications of such peptides requires the administration to a patient of a larger amount of a peptide than if all of the peptide could bind directly to the MHC molecule. The peptide of the present invention overcomes this problem by being

able to bind directly to MHC molecules without further proteolysis or other processing, and so we find that in the absence of proteases, it is actually over a million-fold more active than  
5 previously described peptides. Even in the presence of serum containing proteases that can process longer peptides, the new invention is still about 10-fold more active than such peptides.

The invention comprises a set of synthetic  
10 peptides corresponding to residues 318-327 of HIV-1 strain IIIB gp160 envelope protein in the numbering scheme of Ratner et al. (25), comprising amino acids RGPGRAFVTI, that we have shown to be highly potent for inducing a cytotoxic T cell response to  
15 the HIV-1 envelope protein, and that we have shown does not need processing by proteases. The invention also comprises peptides corresponding to the homologous residues, of other HIV isolates of HIV-1 such as but not limited to the MN isolate  
20 (sequence IGPGRAFYTT). In this context, "homologous" is defined as the region similar in amino acid sequence and in function in the V3 loop of HIV-I gp160. It includes use of the peptides for immunization in any vehicle, adjuvant, route of  
25 administration, or in combination with other material to elicit T-cell immunity, whether for prophylaxis or for immunotherapy of AIDS virus infection.

The general embodiment of the invention is the  
30 presentation of a therapeutic peptide to elicit an immune response. It has been found that such peptides are susceptible to degradative processing by proteolytic enzymes. This has either an activating effect, if a large precursor peptide is  
35 processed to a smaller, more active product, or a

deactivating (inhibitory) effect if a correctly sized peptide is degraded to a small, inactive product. Set forth below are experiments which utilize specific peptides, proteolytic enzymes and inhibitors to assess these processes. These experiments are generalizable in that one may utilize the techniques set forth to examine these processes for any peptide, protease, and inhibitor combination.

Example 2 describes the details of how to assess whether a peptide is a substrate for a proteolytic enzyme by separation of the products of the peptide-protease reaction using High Performance Liquid Chromatography. The isolated products can then be tested for biological activity, if desired, and sequenced to identify any interesting products. Addition of protease inhibitors to the reaction before HPLC separation allows testing for an effective inhibitor. Use of inhibitors specific for particular proteases allows tentative identification of the active protease present in a mixture of proteases.

Example 3 shows a specific example of how a biological assay, rather than HPLC separation, can be used to provide similar information. In Example 3, captopril is used to identify angiotensin converting enzyme as the protease which processes the large MHC class I-binding peptide p18 to the active form p18-I-10. Clearly, different bioassays would be used to assess different sorts of endpoint activities for the peptides, but the general principal illustrated by the experiment remains valid.

The following experimental examples are set forth to illustrate the preferred embodiments of



the present invention.

Upon study of these examples, various modifications of the details of the invention will be apparent to one skilled in the art. Such modifications are intended to be within the scope of the invention.

#### GENERAL METHODS

Mice. H-2-congenic mice on the B10 background and BALB/c mice were purchased from the Jackson Laboratory, Bar Harbor, ME, provided by Drs. D.H. Sachs and R.H. Schwartz of the National Cancer Institute, Bethesda, MD, or bred in our own colony at Biocon, Inc, Rockville, MD. Mice used were 6-18 weeks old.

Recombinant Vaccinia Viruses. vSC-8 (recombinant vaccinia virus containing the Escherichia coli lacZ gene), and vSC-25 (recombinant vaccinia virus expressing the HIV-1 IIIB gp160 envelope glycoprotein without other structural or regulatory proteins of HIV), generous gifts of Dr. Bernard Moss, NIAID, NIH, have been described (47) and were used for immunizing the mice to induce HIV envelope specific CTL.

Peptide Synthesis and Purification. Peptides T1, P18, and HP53 were prepared under GMP conditions by Peninsula Labs, (Belmont, CA) and were single peaks by reverse phase (C18) HPLC in 2 solvents systems. Other peptides were prepared by the multiple simultaneous peptide method of solid-phase peptide synthesis, in polypropylene mesh "tea-bags" as described (48). Peptides were desalted by reverse-phase chromatography on C18 Sep-Pak columns (Waters Associates, Milford, MA), and analyzed by HPLC. Some peptides were prepared by an automated peptide synthesizer (model 430A;

Applied Biosystems, Inc., Foster City, CA) and purified by HPLC.

CTL Generation. Mice were immunized intravenously with  $10^7$  PFU of recombinant vaccinia virus. 4-6wk later, immune spleen cells ( $5 \times 10^6$ /ml in 24-well culture plates in complete T cell medium (CTM; 1:1 mixture of RPMI 1640 and EHAA medium containing 10% FCS, 2mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and  $5 \times 10^{-5}$ M 2-ME) were restimulated for 6d in vitro with peptides and 10% Con A supernatant-containing medium (rat T cell Monoclonal; Collaborative Research, Inc., Bedford, MA). Long-term CTL lines were also generated by repetitive stimulation of immune cells with peptide-pulsed irradiated syngenic spleen cells ( $2.5 \times 10^6$  cells/ml; spleen cells were pulsed with peptides at 1-10  $\mu$ M for 4 h and then irradiated) in 10% rat Con A supernatant-containing medium.

CTL Assay. Cytolytic activity of in vitro secondary CTL or CTL lines was measured as previously described (37,49) using a 6-h assay with  $^{51}\text{Cr}$ -labelled targets, as indicated in the legends. For testing peptide specificity of CTL, effectors and  $^{51}\text{Cr}$ -labeled targets were mixed with various concentrations of peptide, or effectors were cocultured with peptide-pulsed targets. The percent specific  $^{51}\text{Cr}$  release was calculated as  $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$ . Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton-X 100. Spontaneous release was determined from targets cells incubated without added effector cells. The 18Neo ( $\text{H-2}^d$ ; class I MHC+, class II

MHC-neomycin-resistance gene transfected 3T3 fibroblast (37)), L cell (L28; H2<sup>k</sup>), EL4 thymoma cell (H-2<sup>b</sup>), and Con A blasts (other haplotypes) were used as targets.

5     **EXAMPLE 1: TESTING THE EFFECT OF PEPTIDE LENGTH ON**  
      **BINDING TO MHC PROTEINS**

      We investigated the effect of peptide length on functional binding to class I MHC molecules by presenting p18 peptide truncations to plate bound  
10   H-2D<sup>d</sup> in the presence of BSA. A series of shorter peptides contained within p18 were compared with p18 for the ability to stimulate the growth inhibition of the CTL hybridoma B4.2.3 in BSA solution in the absence of serum.

15       In this experiment, 0.2 µg per well soluble H-2D<sup>d</sup> protein was coated (13) onto Immulon 4 plates (Dynatech) which were washed and blocked. The sequence of p18 is RIQRGPGRAFTIGK and of p18-I-10 is RGPGRFAFTI. The sequence of p18-I-9 is  
20   GPGRAFTI, p18-T-9 has the sequence RGPGRFAFT; these two peptides represent the two 9 amino acid overlaps contained within p18-I-10. The two other 9 and 10 residue peptides overlapping p-I-10 that were used were p18-V-9 (QRGPGRAFTV) and p18-V-10  
25   (IRQRGPGRFAFTV). The peptides are named for the last amino acid residue and the length. Peptide and human β2-microglobulin (Calbiochem) (0.2 µg per well) were added to the incubation medium, 0.5% BSA (Sigma fraction V) was added to give a final volume  
30   of 200 µl per well and the plates were incubated at 37°C and 7.5% CO<sub>2</sub> for 22-26 h. The plates were then washed twice with PBS and 2 x 10<sup>4</sup> B4.2.3 T-hybridoma cells added per well in DMEM complete media (13). The plates were incubated from 16-20

h at 37°C and 7.5% CO<sub>2</sub>, then pulsed with 1  $\mu$ Ci [<sup>3</sup>H]thymidine (ICN) and collected 4-8 h later for counting the amount of incorporated label to evaluate growth inhibition (18).

5 In the absence of serum, only the peptide p18-I-10 inhibited the growth of the CTL hybridoma, except at the highest concentration (Figure 1). Therefore, p18-I-10 is much more than 1000-fold more potent than any of the other shorter peptides, and because the two 9-residue peptides contained within it have much less activity, if any, p18-I-10 is the shortest peptide with optimal activity. This result was completely unanticipated.

10 Next, we compared the two peptides of differing lengths for their functional binding to class I MHC molecules in the presence of BSA, ovalbumin, or FCS (see FIG. 2). This binding was evaluated through activation of the B4.2.3 p18 specific T-cell hybridoma, measured by growth inhibition (18) as described above, except that comparison experiments using 0.5% ovalbumin (Sigma grade V), or FCS (Hyclone) were performed alongside the experiment run in BSA. Results are expressed as c.p.m.  $\pm$  s.e.m. of duplicate samples. In control experiments wherein no peptide was added, the following results were obtained: no peptide in BSA, 475,500 c.p.m.  $\pm$  5710 s.e.m.; no peptide in ovalbumin, 512,800 c.p.m.  $\pm$  34,400 s.e.m.; no peptide in FCS 509,900 c.p.m.  $\pm$  3530 s.e.m. With p18, FCS or ovalbumin was required for significant activation of B4.2.3. In contrast, this activation was decreased by FCS or ovalbumin when p18-I-10 was used. The concentration of p18-I-10 which gave half-maximal stimulation was 10<sup>-11</sup> M when added in BSA. This concentration was 10 to 10<sup>2</sup>-fold less

than the half-maximal concentration of p18-I-10 used in FCS and  $10^3$ -fold lower than the half-maximal concentration of p18-I-10 used in ovalbumin. It was more than  $10^6$ -fold lower than the half-maximal concentration of p18 used in BSA.

EXAMPLE 2: ANALYSIS OF PROTEOLYTIC ACTIVATION OF  
PEPTIDE p18 BY SERUM AND BSA

One likely explanation of the results observed in Example 1 is that proteolytic enzymes in ovalbumin and FCS degrade the p18 15-mer to a smaller active form and reduce the active p18-I-10 10-mer to an inactive form. To evaluate this hypothesis we incubated p18 with either ovalbumin or BSA overnight, size fractionated the small MW peptides away from the ovalbumin or BSA, and analyzed them by reverse phase HPLC (FIG 3).

40  $\mu$ l of 2.5 mM p18 was added to 160  $\mu$ l 1% ovalbumin or 1% BSA for 15 h at 37°C. 100  $\mu$ l of each sample was spun through a Centricon 3 filter (Amicon) into 100  $\mu$ l of 1% BSA. The samples were injected into a 4.6 mm X 300 mm C18 reverse phase column (Pharmacia), and eluted with a gradient of 15-30% acetonitrile over 30 minutes at a flow rate of 1 ml per min. 40 1 ml fractions were collected, dried down in a Spin-Vac and resuspended in 200  $\mu$ l dionized water. 25  $\mu$ l of fractions 1-4, 4-8, 9-12, 31-35, and 36-40 were pooled and brought to 200  $\mu$ l with 0.1% BSA. 25  $\mu$ l of the remaining fractions were brought to 200  $\mu$ l with 0.1% BSA. The pooled and unpooled fractions were filter-sterilized and added to H-2D<sup>d</sup> (0.1  $\mu$ g per well) coated plates at dilutions of 1:5 and 1:25 in the presence of 0.2  $\mu$ g per well human  $\beta$ 2-microglobulin. After overnight incubation, the plates were washed and B4.2.3 T-

hybridoma cells added and assayed for growth inhibition as in Example 1. Unpulsed and p18-I-10 pulsed H-2D<sup>d</sup> were included as controls. (In FIG. 3a) B4.2.3 incorporated 345,000 c.p.m. with H-2D<sup>d</sup> incubated with 0.5% BSA. B4.2.3 incorporated 11,900 c.p.m. with H-2D<sup>d</sup> incubated with 0.05  $\mu$ M 18-1-10 in 0.5% BSA. (In FIG. 3b) B4.2.3 incorporated 384,000 c.p.m. with H-2D<sup>d</sup> incubated with 0.5% BSA. B4.2.3 incorporated 9649 c.p.m. with H-2D<sup>d</sup> incubated with 0.05  $\mu$ M 18-1-10 in 0.5% BSA.

A decrease in the amount and a slight increase of the retention time of the major peak of p18 in PBS was seen in the ovalbumin-treated peptide but not in the BSA-treated peptide. The HPLC profile of the ovalbumin-treated p18 also differed from the BSA-treated p18 in amount and retention times of several minor peaks. To determine in which fractions of the ovalbumin-treated p18 the T-cell stimulatory activity eluted, the fractions were assayed for presentation by plate-bound H-2D<sup>d</sup>. The active growth-inhibiting material was in fractions 26 and 27, eluting later than the p18 major peak; these fractions had very little 220 nm absorbance. The BSA-treated p18 fractions were unable to inhibit the growth of the T-cell hybridoma. This observation suggested that a very small proportion of the FCS or ovalbumin processed p18 was a highly active peptide as has been noted for the SV12 peptide and its synthetic contaminants (9). However, in contrast to the latter case, this active peptide is not a contaminant of the original p18 preparation. The active fractions were pooled. Fractions recovered from the HPLC were subjected to automated Edman degradation on an Applied

Biosystems model 470A sequenator, and fractions were identified by amino acid analysis on a model 120A PTH analyzer. FCS treatment of p18 generated similar changes in the HPLC profile (active fractions 24-26), but a clear sequence was difficult to obtain, probably due to a more complex proteolytic system and contaminating serum peptides. The reverse phase HPLC profile of untreated p18 is similar to the profile of BSA treated p18 (major peak BSA-p18 elutes at 19.5 min; major peak p18 elutes at 19.7 min; major peak OVA-p18 elutes at 20.3 min.). The sequence of the active peptide was determined to be XIQRGPGRAFVTI, which is identical to p18 lacking two C-terminal residues and possessing the same C-terminus as p18-1-10. The activity in the ovalbumin appeared to be that of a carboxypeptidase, removing the two C-terminal residues from p18.

EXAMPLE 3: DETERMINATION OF THE CARBOXYPEPTIDASE  
ACTIVITY IN SERUM WHICH ACTIVATES PEPTIDE p18

To identify the carboxypeptidase that processes p18 in FCS, we titrated four carboxypeptidase inhibitors into p18 FCS mixtures, adding them to plate-bound H-2D<sup>d</sup>. The inhibitors used were potato carboxypeptidase inhibitor (19) which blocks tissue carboxypeptidases A and B, Plummer's inhibitor (20) which blocks carboxypeptidase N (serum carboxypeptidase B), captopril (21) which blocks angiotensin converting enzyme (ACE or peptidyl dipeptidase A), and E-64 (22) which blocks cathepsin B (peptidyl dipeptidase B).

The carboxypeptidase inhibitors were titrated as shown in FIG 4a.. An Immulon 4 plate with 0.25

5  $\mu\text{g}$  per well H-2D<sup>d</sup>. 0.2  $\mu\text{g}$  per well human  $\beta 2$ -microglobulin and p18 to give a final concentration of 1  $\mu\text{M}$  were added. The incubation medium was 0.5% FCS. After an overnight incubation B4.2.3 T-hybridoma cells were added and growth inhibition assessed as described in Example 1. Captopril (Sigma) and potato carboxypeptidase inhibitor (Calbiochem) were dissolved in PBS. Plummer's inhibitor (Calbiochem) was dissolved in acidified  
10 deionized water. E-64 (Calbiochem) was dissolved in 33% DMSO (< 1.7% DMSO at highest concentration in experimental wells). The experiment was done in triplicate and results are shown  $\pm$  s.e.m.. In control experiments, B4.2.3 thymidine incorporation  
15 was measured in the absence of peptide and in the absence of inhibitors: no peptide in FCS 145,000 c.p.m.  $\pm$  11,200 s.e.m.; 1  $\mu\text{M}$  p18 in FCS 27,800 c.p.m.  $\pm$  4,400 s.e.m.; no peptide in BSA 145,000 c.p.m.  $\pm$  7000 s.e.m.; 1  $\mu\text{M}$  p18 in BSA 146,000 c.p.m.  $\pm$  1,600 s.e.m.

20 In the presence of FCS, nanomolar concentrations of captopril blocked p18 dependent stimulation of B4.2.3. (FIG 4a). The blocking of FCS processing of p18 occurred at captopril  
25 concentrations  $10^4$  to  $10^5$ -fold lower than that of any of the other carboxypeptidase inhibitors. This result suggested ACE (23, 24) as a major serum processor of p18. Thus, we attempted to process p18 in the absence of serum or ovalbumin using rabbit  
30 lung ACE (FIG. 4b).

Carboxypeptidase N (Calbiochem) and ACE (Sigma) were diluted in PBS and titrated as shown in an Immulon 4 plate with 0.1  $\mu\text{g}$  per well H-2D<sup>d</sup>. The incubation media was 0.5% BSA. After an  
35 overnight incubation B4.2.3 T-hybridoma cells were



added and growth inhibition assessed as in Example 1. The experiment was done in triplicate and results are shown  $\pm$  s.e.m.. The results of the control experiments were: B4.2.3 thymidine incorporation without carboxypeptidases: No peptide in FCS 429,600 c.p.m.  $\pm$  21,000 s.e.m.; No peptide in BSA 411,800 c.p.m.  $\pm$  29,200 s.e.m.; 1  $\mu$ M p18 in FCS 112,600  $\pm$  13,200 s.e.m.; 1  $\mu$ M p18 in BSA 449,600 c.p.m.  $\pm$  21,600 s.e.m.; 0.1  $\mu$ M p18-I-10 in FCS 13,100 c.p.m.  $\pm$  1,000 s.e.m.; 0.1  $\mu$ M p18-I-10 in BSA 5,500 c.p.m.  $\pm$  120 s.e.m.

The purified ACE was able to process p18 without serum, whereas human carboxypeptidase N was unable to do so. ACE was not required for T-cell hybridoma stimulation by p18-I-10 and had some inhibitory effect at high concentrations using both p18 and p18-I-10.

#### EXAMPLE 4: THE INFLUENCE OF ACE PROCESSING ON THE BINDING OF p18 TO ANTIGEN-PRESENTING CELLS

An experiment was performed to evaluate whether the role of ACE in processing p18 observed in the cell-free system described in Examples 1 - 3 would also apply to cell-surface class I molecules. This experiment was done using H-2D<sup>d</sup> transfected L-cells as the antigen presenting cells. In this experiment hybridoma stimulation is indicated by increased thymidine incorporation of the CTLL-2 cells as opposed to decreased thymidine incorporation of the hybridoma cells themselves used to indicate stimulation in the prior experiments.

96-well tissue culture plates (Costar) were blocked with DMEM complete medium. Captopril, Plummer's inhibitor, or no carboxypeptidase

inhibitor was added to give a final concentration of  $10^{-5}$  M and peptide (p18 or P18-I-10) was titrated.  $10^4$  B4.2.3 T-hybridoma cells and  $2 \times 10^4$  H-2D<sup>d</sup> positive L-cells were added to each well. 5 After an overnight incubation, 50  $\mu$ l per well of supernatant was harvested and freeze-thawed. These supernatants were added to  $4 \times 10^3$  CTLL-2 cells in RPMI complete medium to give a final volume of 200  $\mu$ l per well. After an 18 h incubation 10  $\mu$ Ci [<sup>3</sup>H]thymidine was added to each well. 4 h later the CTLL-2 cells were collected and counted for incorporated thymidine. Results are shown as triplicates  $\pm$  s.e.m.

CTLL-2 thymidine incorporation in the absence 15 of peptide was <600 c.p.m.. Peptide p18 and p18-I-10 were titrated in the presence of  $10^{-5}$  M captopril or Plummer's inhibitor in the presence of medium containing FCS (FIG. 5a). Lymphokine production was used to assess T-cell stimulation in 20 the following experiments, avoiding confusion from thymidine uptake by the presenting cells in evaluating T-cell growth inhibition. The p18 concentration required for half-maximal lymphokine production by the hybridoma was increased by  $10^3$  to 25  $10^4$ -fold in the presence of captopril. In contrast, stimulation by p18-I-10 was completely insensitive to inhibition by captopril.

To assess the role of ACE in peptide activation when the antigen is endogenous to the 30 cell, the stimulation of the B4.2.3 hybridoma when both the gp160 and H-2D<sup>d</sup> are expressed by a transfected fibroblast was tested.

96-well tissue culture plates (Costar) were blocked with DMEM complete medium as before. 35 Captopril, Plummer's inhibitor, or no

carboxypeptidase inhibitor was added to give a final concentration of  $10^{-5}$  M and gp160 transfected H-2D<sup>d</sup> positive 3T3 cells were titrated. Neomycin resistance gene transfected 3T3 cells were titrated as a negative control. 10<sup>4</sup> B4.2.3 cells in DMEM complete media were added per well. After an overnight incubation the supernatants were freeze-thawed and added to CTLL-2 cells as described above in this example. Results are shown as triplicates  $\pm$  s.e.m. Captopril had no significant effect on stimulation by p18-I-10, excluding a direct cellular effect of captopril, and Plummer's inhibitor and E-64 had no effect on p18. In contrast, stimulation of the B4.2.3 hybridoma is not affected by captopril or Plummer's inhibitor when a transfectant cell (10) that expresses the gp160 envelope protein and H-2D<sup>d</sup> is used as the antigen source (FIG. 5b).

The data in Figure 5a suggests that the ACE extracellular processing demonstrated in the cell-free system is applicable to the cell-surface system. The data in Figure 5b suggests that the intracellular processing of the antigen is not dependent on an ACE-like activity or occurs in a cellular compartment inaccessible to captopril.

#### EXAMPLE 5: PROTEOLYTIC ACTIVATION OF PEPTIDES DERIVED FROM CYTOMEGALOVIRUS

To demonstrate that the processing of peptide antigens longer than ten residues by a proteolytic clipping mechanism is a general phenomenon, we investigated the activation of peptides derived from murine cytomegalovirus (MCMV).

MCMV variant peptides were titrated in complete medium (containing FCS) in the presence or

absence of  $10^{-5}$  M captopril.  $2 \times 10^4$  H-2L<sup>d</sup> transfected L cells and  $1 \times 10^4$  E1B6 T cell hybridoma cells (anti-H-2L<sup>d</sup> + MCMV) were added per well for an overnight incubation. One  $\mu$ Ci  $^3$ H-thymidine was added per well the following day and 4 hrs. later the cells were harvested and the amount of  $^3$ H-thymidine incorporation was determined. The activation of the E1B6 hybridoma was demonstrated by inhibition of growth.

As shown in figure 6, both MYPHFMPTNL and MYPHFMPTNLG MCMV peptide variants were enhanced in their ability to stimulate a class I-restricted T cell hybridoma by the ACE inhibitor captopril. The activity of the MCMV peptide YPHFMPTNLGK is decreased by an ACE inhibitor in a fashion similar to that observed for peptide p18 as described above. Such a result shows that captopril is useful as an inhibitor of proteolysis of therapeutic peptides. This result also generalizes the effect of ACE to another class I-restricted peptide antigen and clearly demonstrates increased responsiveness of T cells to peptide in the presence of the protease inhibitor captopril.

#### EXAMPLE 6: FINE SPECIFICITY OF p18-SPECIFIC CTL

As previously reported (50), the CTL specific for P18 (18IIIB; HIV-1-IIIB isolate derived) did not crossreactively kill H-2<sup>d</sup> targets infected with recombinant vaccinia virus expressing the envelope gene from the natural HIV-1-RF variant, or targets pulsed with a peptide corresponding to the homologous site in the HIV-1-RF gp160 envelope protein. Therefore, by examining the role of each residue at which these variants differ, we could both identify the residues involved in interaction

with MHC molecule or TCR and also examine the structural basis for the effect of viral variation on T cell reactivity in the several MHC haplotypes. We synthesized a series of peptides with single amino acid substitutions at positions in which 18IIIB (315-329, RIQRGPGRAFTIGK, and 18RF (315-329 $\Delta\Delta$  TKGPGRVIVATGQ where  $\Delta$  indicates a deletion) differ (Fig. 7). Where they were identical an alanine was substituted. Thus, each residue of the 18IIIB sequence was substituted by  $\Delta$ ,  $\Delta$ , T, K, A, A, A, A, V, I, Y, A, T, A, and Q at positions 1 to 15, respectively, to produce peptides 18-1 through 18-15, respectively. The results are presented in Fig. 8 and summarized in Table I.

Table I. The effects of single amino acid substitutions on the recognition of epitopes by P18IIIB-specific CTL lines.

	BALB/c H-2 <sup>d</sup>	B10.D2 H-2 <sup>d</sup>	B10.A H-2 <sup>a</sup>	B10.PL H-2 <sup>u</sup>	B10.P H-2P	B10.Q H-2q
P18(max lysis)	53.5%	54.0%	53.0%	34.5%	36.4%	39.7%
18-1	++	++	++	++	++	++
18-2	++	++	++	++	++	++
18-3	++	++	++	++	++	++
10-4	++	++	++	+	+	++
18-5	++	-	++	++	+	-
18-6	++	++	++	+	++	++
10-7	++	-	++	++	++	++
10-8	+	-	++	+	++	-
18-9	++	-	+	++	++	+
18-10	-	-	-	++	+	++
18-11	+	-	+	++	++	+
18-12	++	++	++	++	+	-
18-13	+	+	++	+	++	-
10-14	++	++	++	++	++	++
18-15	++	++	++	++	++	++

CTL assay was performed in the presence of each peptide at 0.05, 0.5, 1, and 5  $\mu$ M in culture.

++ max lysis  $\geq$  1/2 max lysis with P18.

+ max lysis < 1/2 max lysis with P18 but  $\geq$  15%.

- max lysis < 15%.

Although the summary in Table I of necessity obscures some of the subtle differences between titration curves, it also allows one to discern overall patterns that may be lost in the detailed titrations. As shown previously in the BALB/c strain (50), substitutions at positions 322 (R) (18-8) and 324 (F) (18-10), and 325 (V) (18-11) affected CTL activity. In addition 318 (R) (18-4), 319 (G) (18-5), 320 (P) (18-6), 321 (G) (18-7), 323 (A) (18-9), 326 (T) (18-12), and 327 (I) (18-13) also showed some effect on this CTL line, which was grown by stimulation only with specific peptide 18IIIB but not with the transfectant expressing whole gp160 protein as in the previous study (50). Substitution of 324 (F) with Ile (peptide 18-10) completely abrogated the CTL response of all H-2<sup>d</sup>-restricted CTL (B10.D2, B10.A, and BALB/c mice) and of H-2<sup>P</sup> but not H-2<sup>U</sup> and H-2<sup>Q</sup> CTL. This result indicated that 18-10 can bind to class I MHC molecules of some haplotypes (H-2<sup>U</sup> and <sup>Q</sup>) even though competition studies showed that it did not bind D<sup>d</sup> (50). In B10.D2, using the same class I molecule as BALB/C, the substitution of 319, 321, 322, 323, or 324 completely abrogated the peptide activity. Significant effect was also demonstrated by the substitution of 325, 326, and 327. The reason for the differences from BALB/c is not clear but may reflect difference in the TCR gene repertoire. A few substitutions of the central region of P18 (318, 320, 322, and 327 in B10.PL; 318, 319, 324 and 326 in B10.P) could not sensitize target cells in the B10.PL and B10.P, respectively. Substitutions at 319, 322, 325, 326, and 327 strikingly affected the killing in B10.Q. The

subtle differences between the lines were not likely to be due to heterogeneity of CTL in the lines, because titration of all the peptides with CTL clones from B10.D2 and B10.PL gave virtually identical results. However, substitutions in the N-terminal three positions (315-317) and C-terminal two positions (328-329) had much less effect on killing in any of the strains. Thus, although the details of fine specificity were different, it appeared that CTL of all six strains recognized the same core region, residues 318-327.

To further test this conclusion, and based on other observations described in the preceding Examples, a truncated synthetic peptide was synthesized, 18-I-10, consisting of this 10 residue segment, 318-327, and tested for recognition by CTL lines of all four MHC haplotypes (Fig. 8). This 10-residue core peptide was actually found to be more active than the full-length P18 when presented by all four class I molecules. Thus, the moderate effects of substitutions at positions 315-317 and 328-329 must have been due to other effects of flanking residues on peptide conformation or other aspects of recognition. However, the results shown in Figure 8 clearly indicate that all four class I molecules present the same core 10-residue sequence.

To determine if the same or overlapping sites within P18 are presented with the different class I MHC molecules, we used naturally occurring substitutions within this area, which is in the hypervariable region of gp160. There is no cross-reactive killing between P18IIIB and P18RF. To localize the critical residues of P18 for recognition by CTL of five different MHC



haplotypes, we used 15 substituted peptides, each with a single substitution. There was observed some similarity of fine specificity of CTL lines against P18 restricted by different haplotypes.

5 The substitutions of 322 (R) by Ala and 324 (F) by Ile markedly reduced the CTL recognition of peptide in BALB/c (H-2<sup>d</sup>), and the latter substitution (324) appeared to be critical for other CTL lines restricted by D<sup>d</sup> (B10.D2) and by H-2<sup>P</sup> but not for

10 H-2<sup>u</sup> and H-2<sup>q</sup> restricted CTL lines. In B10.A mice and H-2<sup>d</sup> mice, substitution of residue 325 (V) also strikingly abrogated the activity of P18. The substitutions of 319 and the residues between 321 and 326 were important for P18 to be presented to

15 the CTL line of B10.D2. The difference of fine specificity using these substituted peptides between BALB/c, B10.D2 and B10.A therefore suggests differences in TCR structures of CTL lines restricted by D<sup>d</sup> class I molecules in these

20 strains. P18 may be presented by the D<sup>d</sup> class I MHC molecules to different CTL in a very similar manner, or alternatively, it is possible that the peptide can bind in more than one way to the same MHC molecule (51). In either case, CTL with

25 different TCR would be differentially sensitive to the different substitutions. Although the fine specificity was different from strain to strain, the activity of P18 was less affected by the substitution of the three N-terminal residues

30 (315-317) and the two C-terminal residues (328-329) than the central 10 residues in all six strains. Definition of this 10-residue core was confirmed using a 10-residue peptide, 18-I-10, which was more active than the whole P18 in for recognition by CTL

35 with all four class I molecules. Thus, the

different MHC molecules are not simply seeing different adjacent or partially overlapping antigenic determinants within the same peptide. As in the case of HP53, the requirement for the same  
5 core region for presentation of P18 by multiple class I MHC molecules indicates that this is a single broadly presented antigenic site and may make this peptide valuable for vaccine development in a broadly MHC diverse population. It also  
10 suggests that these core regions of these two peptides have a predilection to bind to class I MHC molecules in general, accounting for the widespread recognition of these peptides.

15 EXAMPLE 7: SURVEY OF MHC CLASS I MOLECULES PRESENTING SPECIFIC PEPTIDES TO CTL LINES IN H-2<sup>d</sup> STRAINS

Based on the experimental data using L cell transfectants expressing D<sup>d</sup>/L<sup>d</sup> class I molecules, a previous study (50) demonstrated that P18 is seen  
20 in H-2<sup>d</sup> mice only with the class I molecule D<sup>d</sup> and that the  $\alpha 1$  and  $\alpha 2$  domains of D<sup>d</sup> were both necessary and sufficient in the context of an intact class I molecule. In this study, we used transfectants expressing K<sup>d</sup>, D<sup>d</sup>, or L<sup>d</sup> molecules to  
25 determine which molecule was specifically required for the presentation of P18 and HP53 in H-2<sup>d</sup> and H-2<sup>a</sup> strains. The transfected gene products expressed on the eight H-2<sup>k</sup> L cell transfectants are shown in Table II. The targets were pulsed  
30 with the indicated peptide and labeled with <sup>51</sup>Cr at same time.

Table II. MHC class I molecules responsible for the presentation of the specific peptides to CTL lines in the H-2d strains.

L cell	transfectant	Class I molecules	α1	α2	α3	TM†	Percent specific lysis			
							BALB/c		B10.D2	
targets							HP53 line P18 line		HP53 line P18line	
							B10.A		HP53 line P18-line	
T4.8.3		Dd	Dd	Dd	Dd	Dd	25.5	44.4	37.8	82.1
T1.1.1		Ld	Ld	Ld	Ld	Ld	0.5	-0.5	2.7	-1.0
B4III2		Kd	Kd	Kd	Kd	Kd	3.5	10.3	3.5	4.1
DMT26.5S1		-	Dd	Dd	Dd	Dd	1.6	-1.2	3.4	-2.3
T37.2.1		Dd	Dd	Ld	Ld	Ld	21.0	34.7	30.8	51.0
T37.1.3		Ld	Ld	Dd	Dd	Dd	1.5	2.5	2.3	2.6
T9.10.3		Dd	Ld	Ld	Ld	Ld	1.0	2.7	1.1	3.8
DMT34.5		Ld	Dd	Dd	Dd	Dd	1.4	1.4	3.0	2.1
L28*		Kk	Kk	Kk	Kk	Kk	1.0	1.6	1.6	1.3
18Neo*		Dd	Dd	Dd	Dd	Dd	53.2	50.0	58.5	71.0
									60.8	47.0

Target cells were pulsed overnight with each specific peptide (10μM of HP53 and 1μM of P18) and washed three times.  
Effector/ Target ratio=5/1.

† TM: transmembrane portion of the Dd molecule.

\* L28; DAP3 L cell (H-2k) transfected with PSV2neo gene alone, 18Neo; BALB/c 3T3 fibroblast (H-2d) transfected with PSV2neo alone.

T37.2.1 ( $\alpha 1\alpha 2$  of  $D^d$ ) and T4.8.3 ( $D^d$ ) were found to present HP53 as well as P18. Any other  $D^d/L^d$  exon-shuffled transfectant targets were not sensitized with these peptides. Therefore both  $\alpha 1$  and  $\alpha 2$  domain were necessary and together sufficient to present these peptides. The  $K^d$  molecule also presented P18 to a very small population of CTL in BALB/c but not in B10.D2 or B10.A mice. In B10.Q, we also used well-defined recombinant mice, B10.AKM ( $H-2^m$ ,  $K^k/D^q$ ) and B10.T(6R) ( $H-2Y^2$ ,  $K^q/D^d$ ) (shown in Table III), to map the restriction element in the  $H-2^q$  haplotype. The results demonstrated that  $D^q$  (or  $L^q$ ) but not  $K^q$  could present both P18 and HP53 to  $H-2^q$  CTL.

Table III. MHC class I molecules responsible for the presentation of HP53 and P18 to CTL lines in B10.Q

Con A	Haplotype	Class I Alleles	Percent specific lysis			
			HP53 line		P18 line	
			Targets pulsed with		Targets pulsed with	
		K D/L	HP53	none	P18	none
B10.Q	q	q g	17.3	0.9	38.7	1.5
B10.AK4	m	k g	19.4	-1.1	51.0	-3.1
B10.T (GR)	y2	q d	3.3	2.2	2.2	4.8
BA1B/c	d	d d	2.8	4.5	3.2	2.4

Target cells were pulsed overnight with 10 $\mu$ M of HP53 and 1 $\mu$ M of P18, and washed three times.  
Effector/ Target ratio=10/1

We were able to map the restriction in the H-2<sup>d</sup> and a and H-2<sup>q</sup> haplotypes to D<sup>d</sup> and D<sup>q</sup> (or L<sup>q</sup>), respectively. Appropriate recombinant mouse strains do not exist to separate D<sup>q</sup> and L<sup>q</sup> in the H-2<sup>q</sup> haplotype or to map the restriction to K or D in the other haplotypes. However because peptides are more frequently presented by more than one allele from the same locus than by MHC molecules of different loci (26,52), it is likely that these peptides are presented by the D molecules of the other haplotypes as well.

To determine whether the response to the immunodominant epitope of the HIV-1-IIIB envelope protein also depends on both the  $\alpha 1$  and  $\alpha 2$  domains of the D<sup>d</sup> class I molecules, we used eight L cell (H-2<sup>k</sup>) transfectants with different exon shuffles between D<sup>d</sup> and L<sup>d</sup>. The results revealed (Table II) that the P18 and HP53 peptides required both  $\alpha 1$  and  $\alpha 2$  domains of the D<sup>d</sup> molecule for effective peptide presentation. We found that a small population of P18 specific CTL derived from BALB/c spleen cells immunized with vSC25 vaccinia virus expressing gp160 could also recognize P18 presented by K<sup>d</sup> class I molecules to some extent. For the presentation of P18 by D<sup>d</sup>, two domains  $\alpha 1$  and  $\alpha 2$  were sufficient and neither the  $\alpha 1$  and  $\alpha 2$  domain alone was sufficient for the presentation, when the other domains derived from L<sup>d</sup>. Therefore, both the  $\alpha 1$  and  $\alpha 2$  domain derived from D<sup>d</sup> are necessary and together sufficient, in the context of an intact class I molecule, for the peptide presentation of P18 and HP53. This can be contrasted with examples of peptides broadly presented by class II molecules in which the presenting element, DR or I-E, has a nonpolymorphic alpha chain and only the beta chain

is polymorphic, so that the permissiveness could depend on interaction primarily with one side of the MHC-peptide-binding groove (44,45).

It is thought that a vaccine eliciting HIV-specific CTL may be protective against HIV, because CTL can block outgrowth of HIV in vitro (53,54). Here it was shown that P18 and HP53 from gp160 were found to be presented by four different class I MHC molecules to CTL as well as to helper T cells by class II MHC as previously shown. The broad recognition of these peptides with different classes of MHC molecules as well as different alleles of class I molecules suggests that these peptides could play a versatile role as components of a vaccine for HIV.

EXAMPLE 8: EFFICACY OF THE REGION OF HIV gp160  
HOMOLOGOUS TO PEPTIDE p18-I-10 IN ACTIVATION OF CTL

Purified D<sup>d</sup> class I MHC molecules were coated onto plastic microtiter wells as described in Example 1, and pulsed with the indicated concentrations of p18MN or p18MN-T-10 (IGPGRAFYTT) in BSA solution in the absence of serum, as in Example 1. Then, instead of the T-cell hybridoma, cells of an HIV-1 MN-specific CTL line (64) were added and cultured overnight. Culture supernatants were then harvested and tested for interferon-gamma production by ELISA as a measure of CTL activation (Figure 9). The results clearly show 1) that p18MN-T-10 is the active core of p18MN, exactly homologous to the p18-I-10 active peptide from p18; and 2) in the absence of serum to process the peptides, pp18MN-T-10 is able to bind to the class I molecule and be presented to CD8<sup>+</sup> cytotoxic T cells, whereas the 15-mer p18MN is not. Thus, the

results with p18-I-10 are generalizable to other strains of HIV-1, such as the MN isolate.

EXAMPLE 9: IL-2 PRODUCTION BY gp160-IMMUNE CD4<sup>+</sup> T  
CELLS STIMULATED BY PEPTIDES p18 AND p18-I-10

5 To demonstrate that peptides p18 and p18-I-10 would be recognized by class II MHC molecules, the following experiment was performed.

Spleen cells from BALB/c mice immunized with recombinant vaccinia virus expressing HIV-1 IIIB  
10 envelope protein gp160 were depleted of CD8<sup>+</sup> T cells with anti-CD8 and complement to remove CTL, so that the only cells responding were CD4<sup>+</sup> helper T cells. They were then stimulated with peptides p18 or p18-I-10 at varying concentrations shown for  
15 24 hrs. at 37° C, and the culture supernatants were then harvested and tested for IL-2 by the ability to stimulate [<sup>3</sup>H]-thymidine incorporation by the IL-2 dependent T-cell line HT2A in another 24-hour culture. (Figure 10). In a control experiment,  
20 the response was abrogated by treatment with anti-CD4 antibody.

The data show that p18-I-10 is as potent as the full-length p18 at stimulating IL-2 production. Therefore, this same 10-residue peptide is not only  
25 much more potent for stimulating CTL, but is also capable of stimulating helper T cells.

EXAMPLE 10: CHEMICAL MODIFICATION OF THE PEPTIDES  
TO ENHANCE THEIR PHARMACOLOGIC CHARACTERISTICS

30 Small peptides circulating in the blood are subject to degradation by proteolytic action and clearing by the kidneys. Yet, a number of naturally occurring peptides are found in the circulation, for example the enkephalins. These



small peptides are often found to be modified by amidation of the carboxy-terminus (55,56). Thus, it may prove advantageous to produce chemically modified variants of the peptides for use in therapeutic applications. The enzymatic carboxy-terminal amidation of a synthetic peptide has been described (58,59). Also, the addition of residues useful for the cross-linking of the peptides to carrier proteins for immunizations or to solid supports for immunoassay or antibody purification applications may prove advantageous. Many means for chemical modification of peptides are well known in the art.

The peptides of the instant invention could also be coupled to, or co-synthesized with, peptides that bind to or induce production of neutralizing antibodies to HIV or helper T-cells specific for HIV. Attachment to HIV specific carriers would cause a memory helper T-cell response on exposure to HIV, in contrast to the use of HIV unrelated carriers which would not produce such a memory response on exposure to the virus. Useful HIV specific carriers are, for example; as described in Cease et al. (41 and U.S. Patent 5,081,226 to Berzofsky et al.), Hale et al. (42 and U.S. Patent 5,030,449 to Berzofsky et al.) and Palker et al. (55), which are hereby incorporated by reference.

EXAMPLE 11: ADMINISTRATION OF PEPTIDES AS A  
VACCINE  
AGAINST HIV

The aim of the research of a large number of biomedical researchers is the production of a vaccine which would produce protection to humans

from infection by HIV or therapeutic benefit in AIDS treatment. The instant invention provides peptides that are useful for the preparation of such vaccines as well as specifying six particular peptides as candidates based on the production of a T-cell response to the protein target from which the peptides are derived in mice immunized with the peptide. A pharmaceutical composition including a vaccine in accordance with the present invention comprises an effective antigenic or therapeutic amount of at least one of the peptides and a pharmaceutically acceptable carrier such as physiological saline, non-toxic, sterile buffer and the like. A therapeutically effective amount of peptide is an amount in the range of 10 to 1000  $\mu$ g of peptide per person, preferably about 100  $\mu$ g. Of course, additives such as preservatives, sterilants, adjuvants and the like, well known to one of ordinary skill in the art, could also be included in the pharmaceutical composition to maintain or increase the efficacy of the preparation.

It is proposed that peptides of the instant invention can also be administered as a vaccine in a fashion similar to that for the administration to primates of a synthetic peptide vaccine against hepatitis B as described by Itoh (60). An alternative method for the preparation of vaccines involves the use of Protein A coated microbeads that bind immune complexes of an antibody and the immunizing antigen on their outer surface described for example in Platt, et al., U.S. patent number 4,493,825, hereby incorporated by reference.

Methods of immunization with peptides to induce CD8<sup>+</sup> cytotoxic T cells which could be used

include those of Aichele et al (61), Deres et al. (62) and Kast et al. (63).

The invention being thus described, it is clear that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

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CLAIMS

What is claimed is:

- 1        1. A polypeptide comprising an amino acid  
2        sequence that is a portion of the HIV envelope  
3        glycoprotein gp160 which elicits an immune  
4        response characterized by response of both MHC  
5        class I-restricted and MHC class-II restricted  
6        elements of the mammalian immune system, said  
7        polypeptide eliciting said response in a solution  
8        at a concentration of peptide of less than  $10^{-6}$   
9        M.
- 1        2. An isolated polypeptide according to  
2        claim 1, wherein said portion of the HIV envelope  
3        glycoprotein gp160 is the region homologous to  
4        amino acids 318-327 of isolate IIIB.
- 1        3. An isolated polypeptide having an amino  
2        acid sequence selected from the group consisting  
3        of: RGPGRFVVTI, IGPGRFYT, IGPGRFYAT,  
4        KGPGRVIYAT, IGPGRFHTT, IGPGRTLIAR, LGPGRVWYTT,  
5        and IGPGRFRTR.
- 1        4. An isolated polypeptide having the amino  
2        acid sequence RGPGRFVVTI.

1           5. An isolated polypeptide having the amino  
2 acid sequence IGPGRAFYTT.

1           6. An isolated polypeptide having the amino  
2 acid sequence IGPGRAFYAT.

1           7. An isolated polypeptide having the amino  
2 acid sequence KGPGRVIYAT.

1           8. An isolated polypeptide having the amino  
2 acid sequence IGPGRAFHTT.

1           9. An isolated polypeptide having the amino  
2 acid sequence IGPGRTLVAR.

1           10. An isolated polypeptide having the amino  
2 acid sequence LGPGRVWYTT.

1           11. An isolated polypeptide having the amino  
2 acid sequence IGPGRAFRTT.

1           12. A pharmaceutical composition comprising  
2 an amount of a peptide according to claim 1 that  
3 is prophylactically or therapeutically effective  
4 in the treatment of HIV infection and a  
5 pharmaceutically acceptable carrier.

1           13. A pharmaceutical composition comprising  
2 an amount of a peptide according to claim 2 which  
3 is prophylactically or therapeutically effective  
4 in the treatment of HIV infection and a  
5 pharmaceutically acceptable carrier.

1           14. A pharmaceutical composition comprising  
2 an amount of a peptide according to claim 3 which



1 is prophylactically or therapeutically effective  
2 in the treatment of HIV infection and a  
3 pharmaceutically acceptable carrier.

1 15. A method of prophylactically or  
2 therapeutically treating HIV infection which  
3 comprises administering the composition of claim  
4 12 to a patient.

1 16. A method of prophylactically or  
2 therapeutically treating HIV infection which  
3 comprises administering the composition of claim  
4 13 to a patient.

1 17. A method of prophylactically or  
2 therapeutically treating HIV infection which  
3 comprises administering the composition of claim  
4 14 to a patient.

1 18. A method according to claim 15, which  
2 further comprises administering, with the  
3 composition, an amount of a protease inhibitor  
4 sufficient to prevent proteolysis of the peptide.

1 19. A method according to claim 16, which  
2 further comprises administering, with the  
3 composition, an amount of a protease inhibitor  
4 sufficient to prevent proteolysis of the peptide.

1 20. A method according to claim 17, which  
2 further comprises administering, with the  
3 composition, an amount of a protease inhibitor  
4 sufficient to prevent proteolysis of the peptide.

1 21. A method according to claim 18, wherein

1 said protease inhibitor is an inhibitor of  
2 angiotensin converting enzyme.

1 22. A method according to claim 19, wherein  
2 said protease inhibitor is an inhibitor of  
3 angiotensin converting enzyme.

1 23. A method according to claim 20, wherein  
2 said protease inhibitor is an inhibitor of  
3 angiotensin converting enzyme.

1 24. A method according to claim 21, wherein  
2 said protease inhibitor is captopril.

1 25. A method according to claim 22, wherein  
2 said protease inhibitor is captopril.

1 26. A method according to claim 23, wherein  
2 said protease inhibitor is captopril.

1 27. A method of use of the protease  
2 inhibitor captopril which comprises administering  
3 to a patient a therapeutically or  
4 prophylactically effective amount of a peptide  
5 together with an amount of captopril sufficient  
6 to prevent proteolysis of the peptide in vivo.

1 28. A method of prophylactically or  
2 therapeutically treating HIV infection, which  
3 comprises administering to a patient an effective  
4 amount of a peptide comprising an amino acid  
5 sequence homologous to the amino acids 315-329 of  
6 the IIIB isolate and also an amount of an  
7 angiotensin converting enzyme sufficient to  
8 activate the peptide by limited proteolysis.

1           29. A method according to claim 28, wherein  
2       said peptide comprises an amino acid sequence  
3       selected from the group consisting of  
4       RIQRGPGRAFYTIGK, RIHIGPGRAFYTTKN,  
5       SITKGPGRVVIYATGQ, SIHIGPGRAFYATGD,  
6       SLSIGPGRAFRTREI, SISIGPGRAFFATTD,  
7       SIYIGPGRAFHTTGR, GIAIGPGRTLYAREK,  
8       RVTLGPGRVWYTTGE, SIRIGPGKVFTAKGG,  
9       GIHFGPGQALYTTGI, STPIGLGQALYTTRG,  
10      STPIGLGQALYTTRI, RTPTGLGQSLYTTRS.

1           30. A method of eliciting a cytotoxic T  
2       lymphocyte response in a mammal which comprises  
3       administering to said mammal a peptide according  
4       to claim 1.

1           31. A method of eliciting a cytotoxic T  
2       lymphocyte response in a mammal which comprises  
3       administering to said mammal a peptide according  
4       to claim 2.

1           32. A method of eliciting a cytotoxic T  
2       lymphocyte response in a mammal which comprises  
3       administering to said mammal a peptide according  
4       to claim 3.

1           33. A method according to claim 30, which  
2       further comprises administering, with the  
3       composition, an amount of a protease inhibitor  
4       sufficient to prevent proteolysis of the peptide.

1           34. A method according to claim 31, which  
2       further comprises administering, with the  
3       composition, an amount of a protease inhibitor  
4       sufficient to prevent proteolysis of the peptide.

1           35. A method according to claim 32, which  
2 further comprises administering, with the  
3 composition, an amount of a protease inhibitor  
4 sufficient to prevent proteolysis of the peptide.

1           36. A method according to claim 33, wherein  
2 said protease inhibitor is an inhibitor of  
3 angiotensin converting enzyme.

1           37. A method according to claim 34, wherein  
2 said protease inhibitor is an inhibitor of  
3 angiotensin converting enzyme.

1           38. A method according to claim 35, wherein  
2 said protease inhibitor is an inhibitor of  
3 angiotensin converting enzyme.

1           39. A method according to claim 36, wherein  
2 said protease inhibitor is captopril.

1           40. A method according to claim 37, wherein  
2 said protease inhibitor is captopril.

1           41. A method according to claim 38, wherein  
2 said protease inhibitor is captopril.

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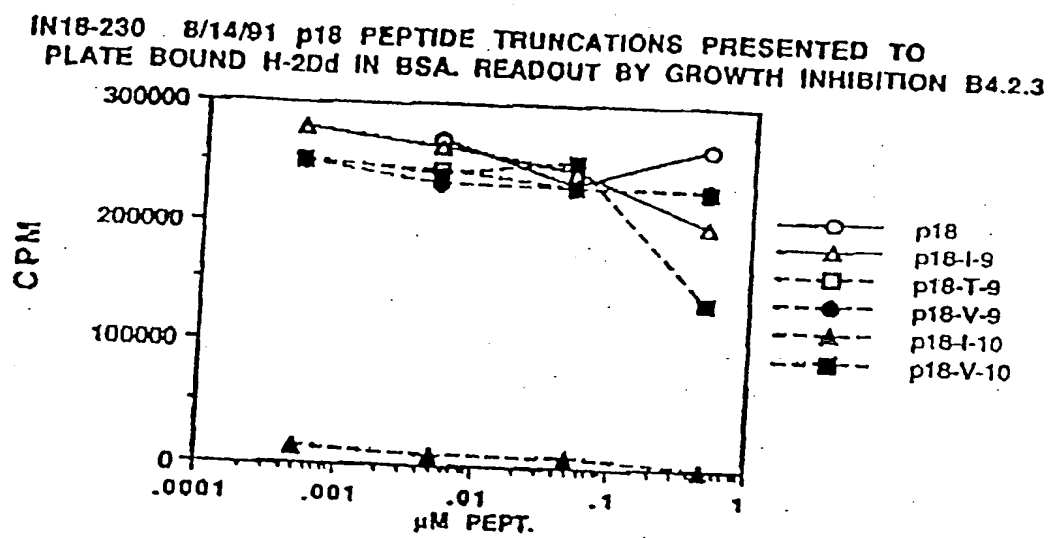


Figure 1.

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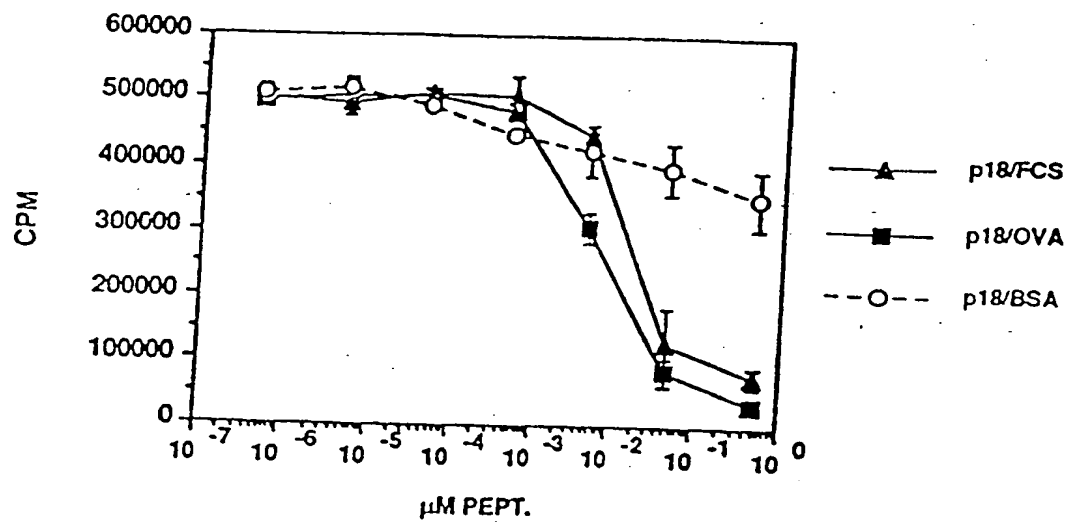


Figure 2 a

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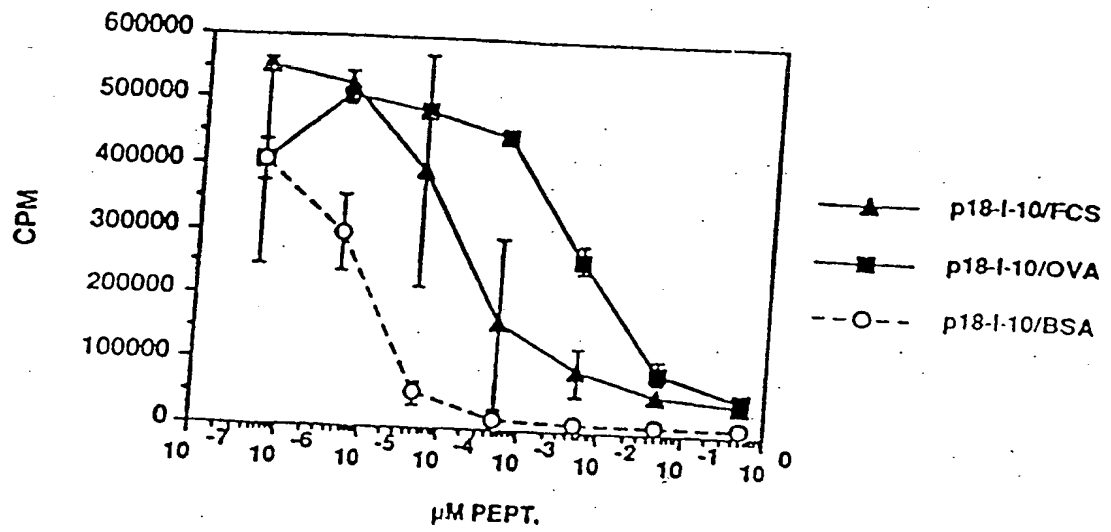


Figure 2b

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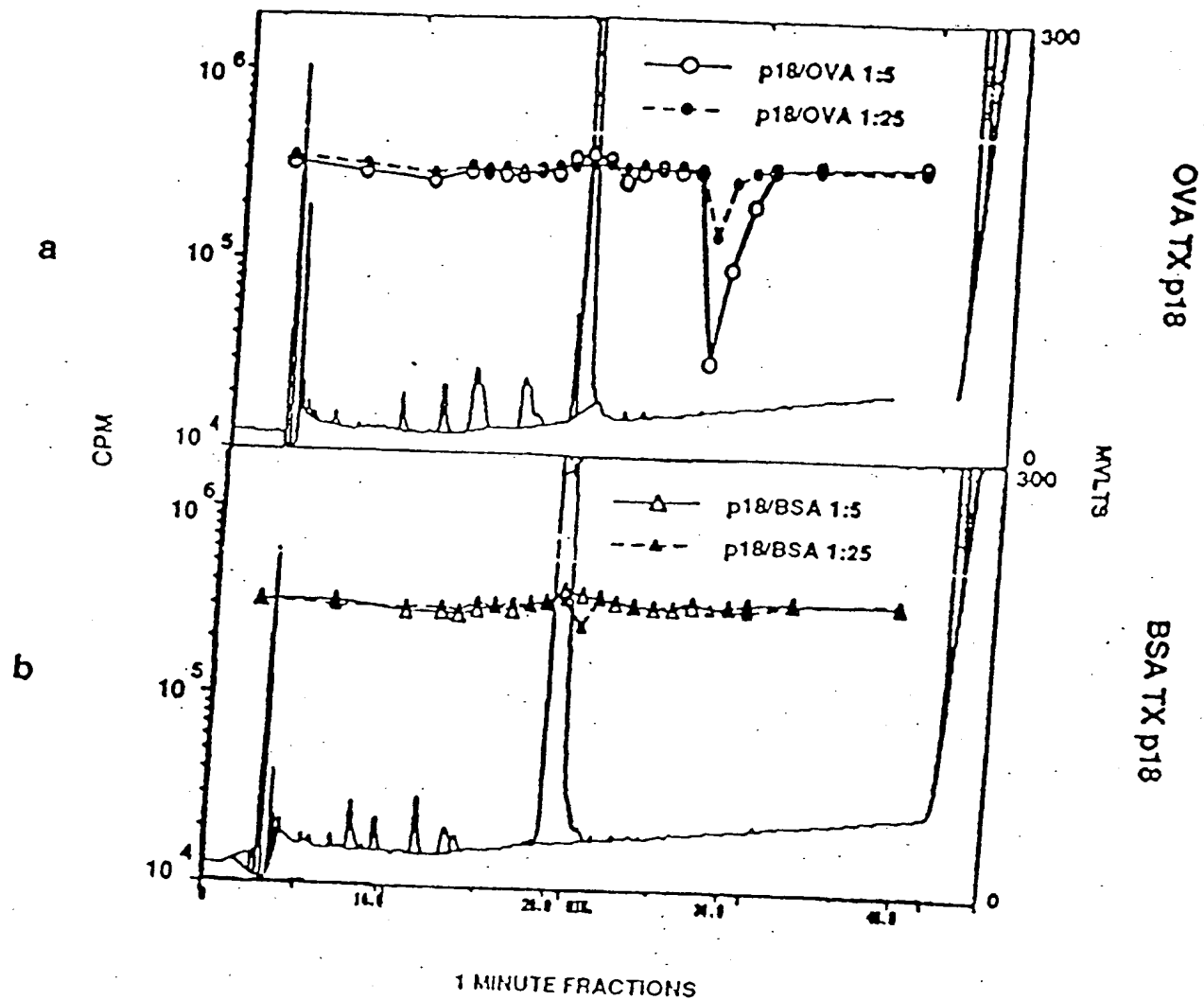


Figure 3



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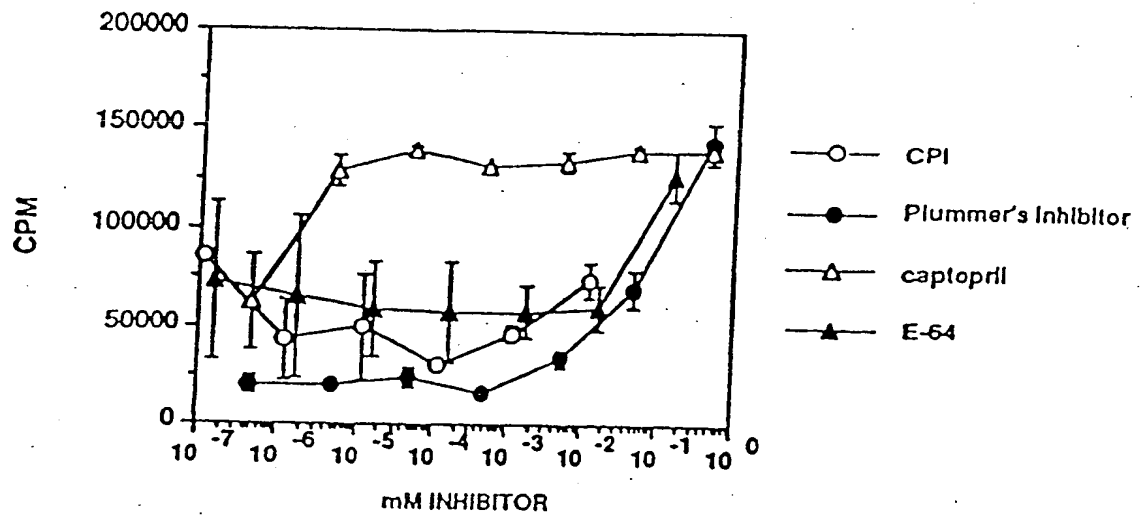


Figure 4a

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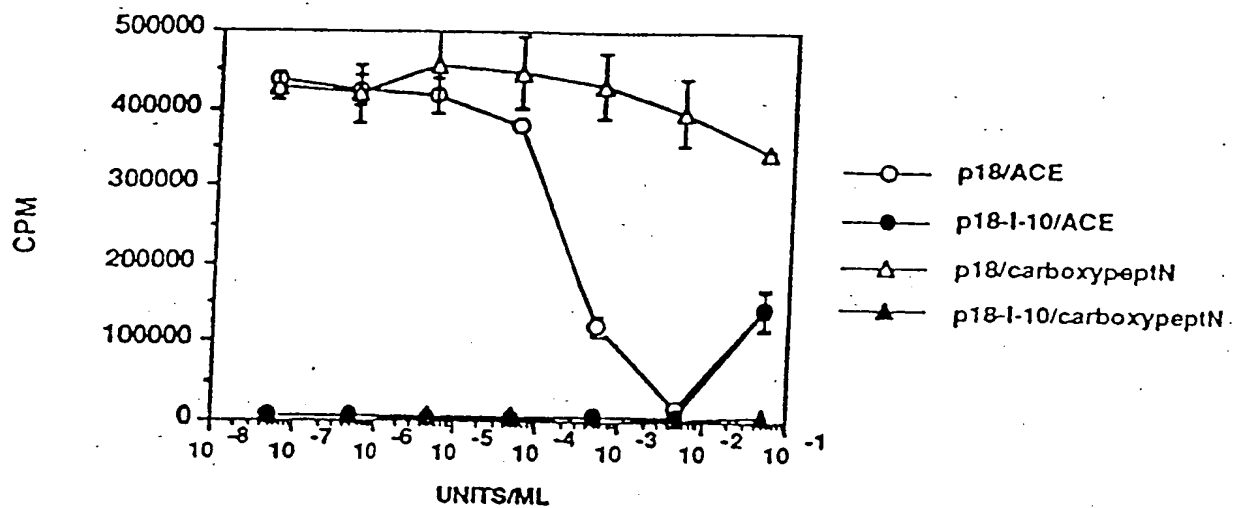


Figure 4b

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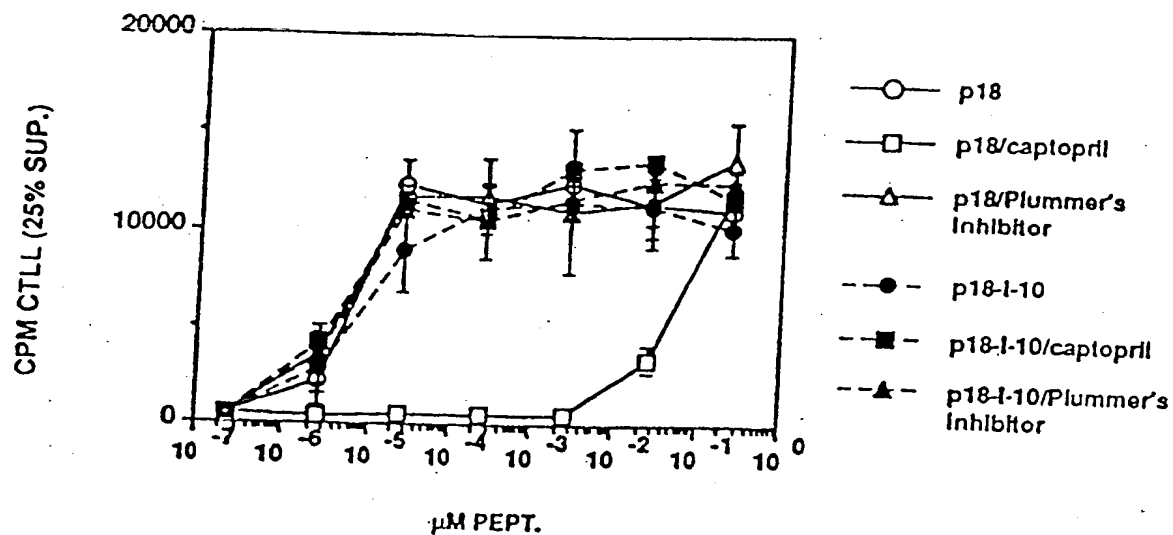


Figure 5a

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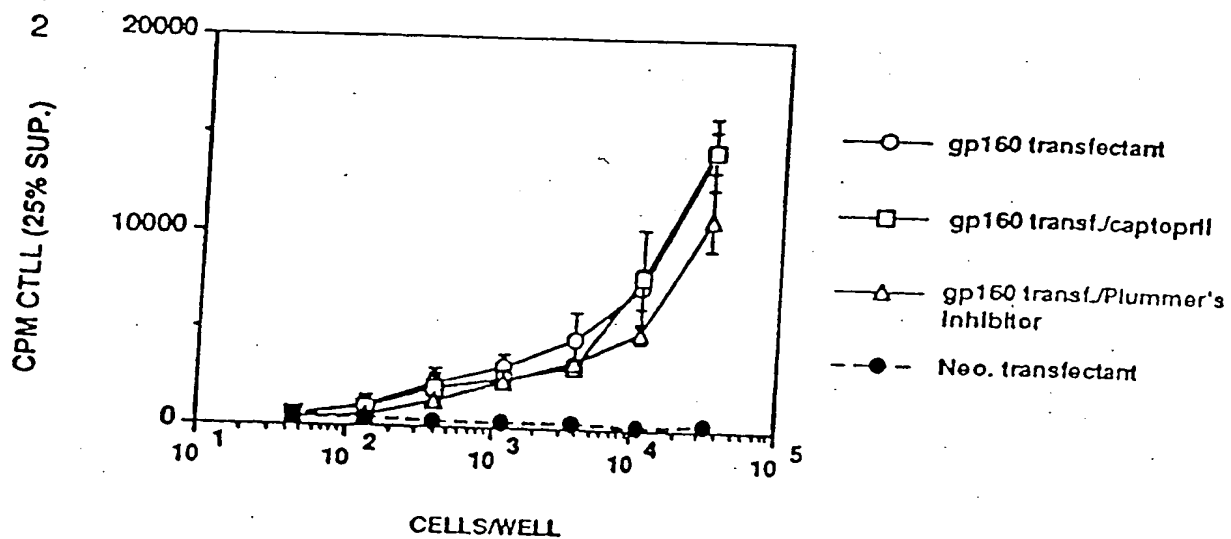


Figure 5b

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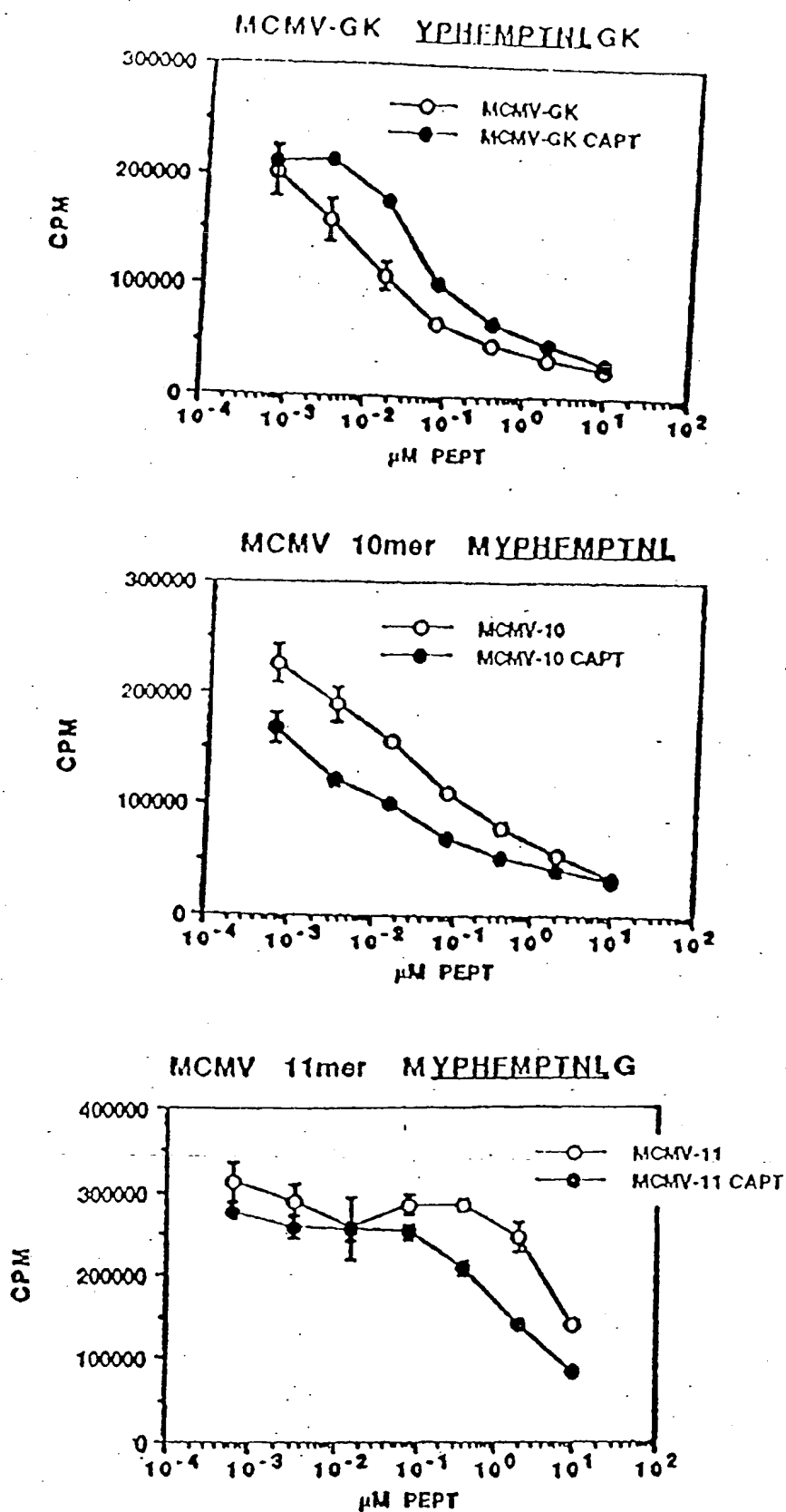


FIGURE 6

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	315														329
18IIIB(P18)	R	I	Q	R	G	P	G	R	A	F	V	T	I	G	K
18RF	Δ	Δ	T	K	G	P	G	R	V	I	Y	A	T	G	Q
18-1	Δ														
18-2		Δ													
18-3			T												
18-4				K											
18-5					A										
18-6						A									
18-7							A								
18-8								A							
18-9									V						
18-10										I					
18-11											Y				
18-12												A			
18-13													T		
18-14														A	
18-15															Q
18I10				R	G	P	G	R	A	F	V	T	I		

Figure 7

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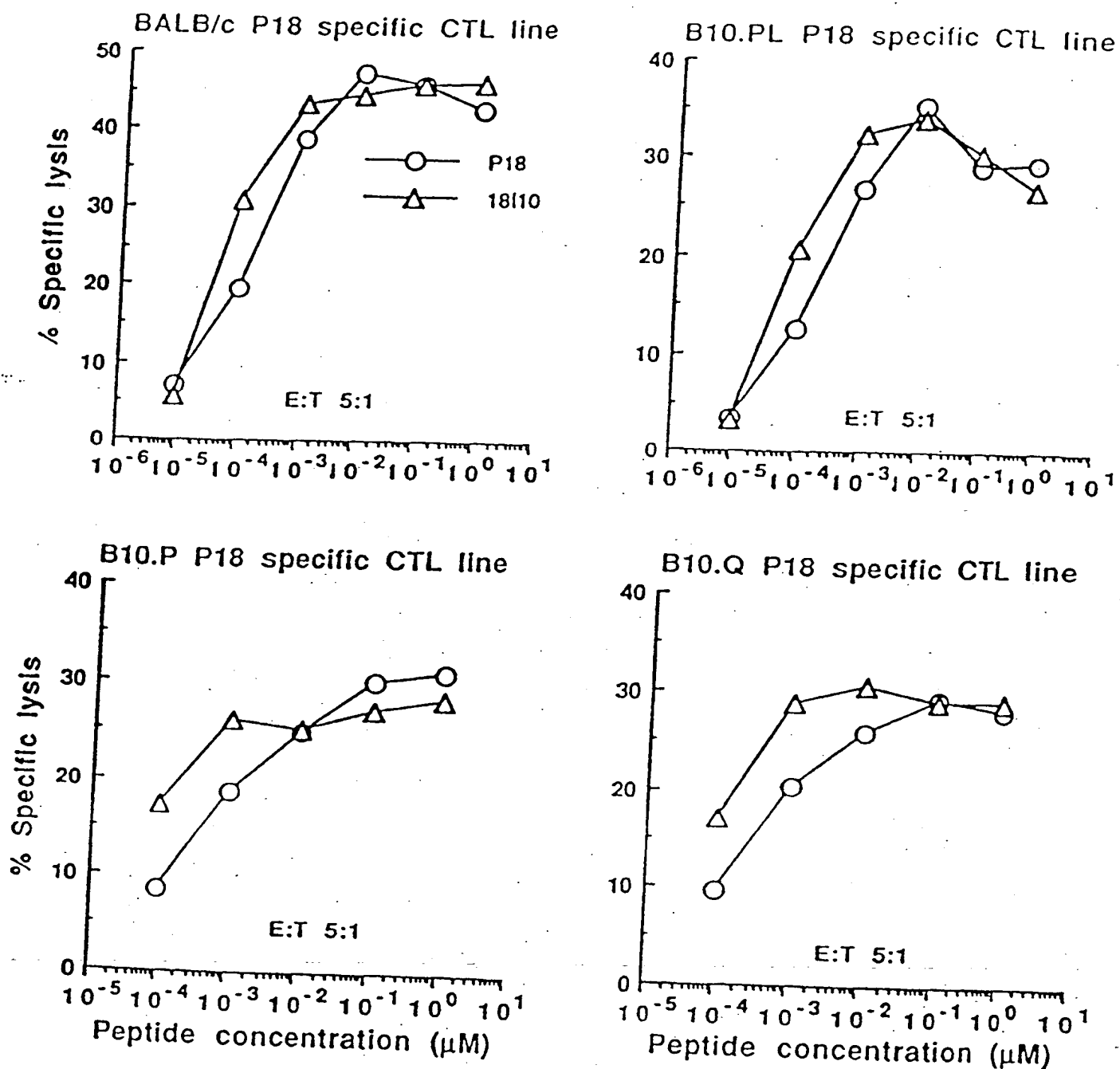


Figure 8

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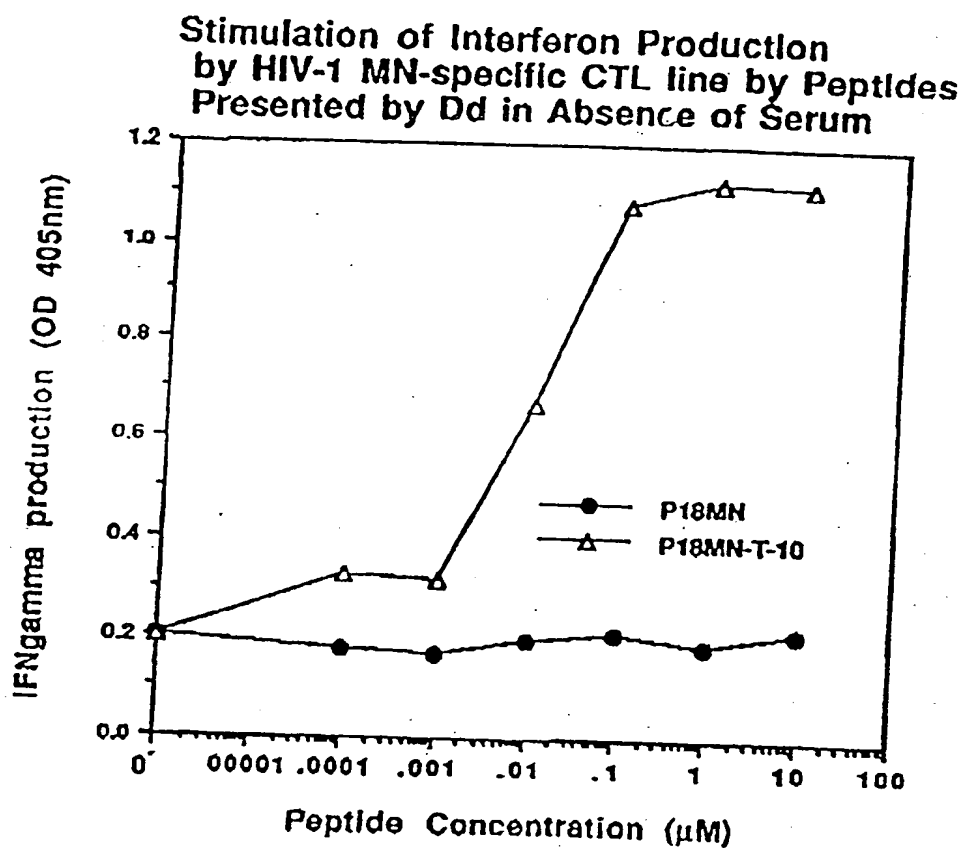


Figure 9



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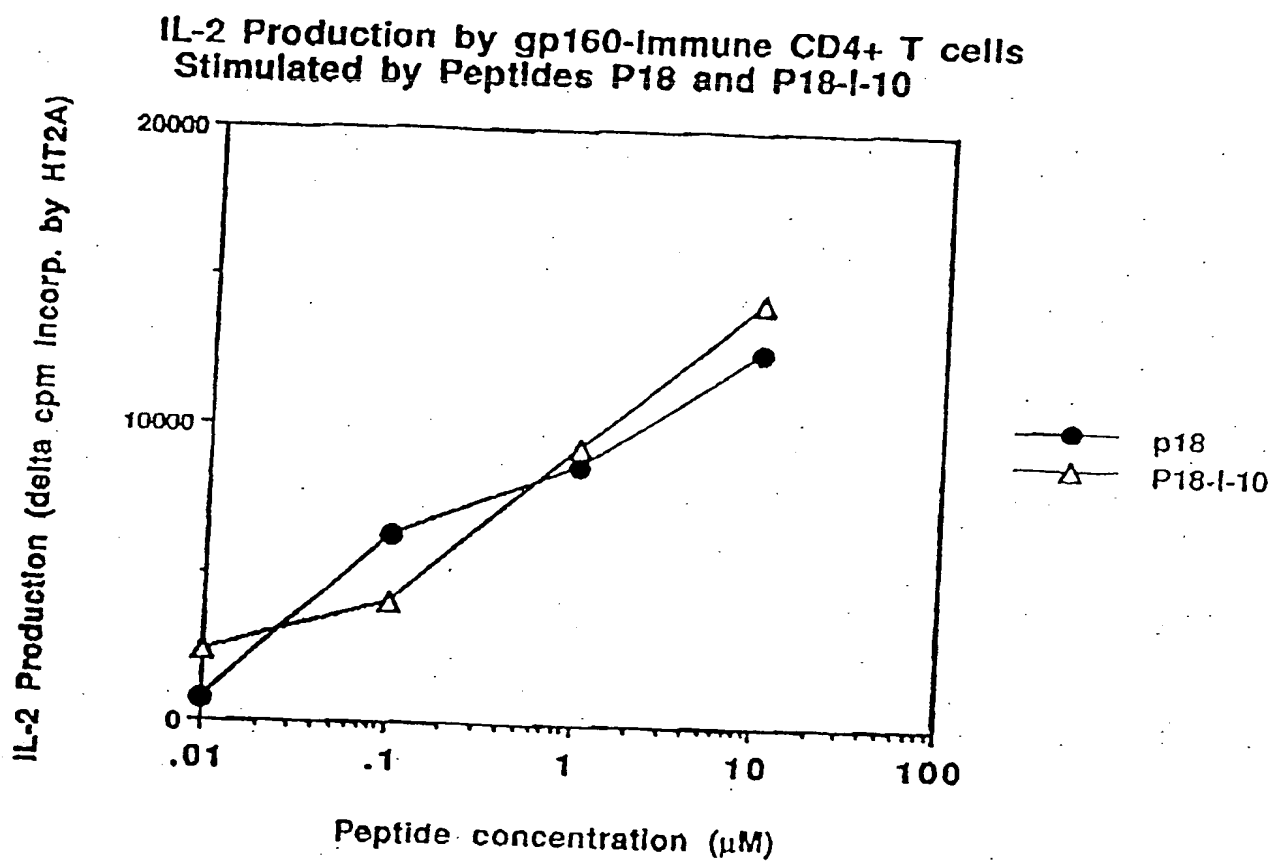


Figure 10



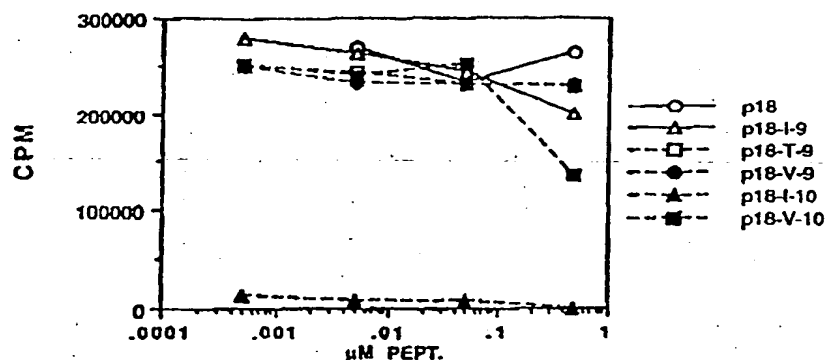


## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 5 :</b> <b>C12N 15/49, C07K 13/00, C12N 15/86</b> <b>A61K 39/21 // (A61K 39/21, 31:40)</b> <b>C07K 15/00</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 93/18055</b> <b>(43) International Publication Date:</b> 16 September 1993 (16.09.93)
<b>(21) International Application Number:</b> PCT/US93/01925 <b>(22) International Filing Date:</b> 8 March 1993 (08.03.93)  <b>(30) Priority data:</b> 847,311 6 March 1992 (06.03.92) US  <b>(71) Applicant:</b> THE GOVERNMENT OF THE UNITED STATES OF AMERICA as represented by THE DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; National Institutes of Health, Box OTT, Bethesda, MD 20892 (US).  <b>(72) Inventors:</b> BERZOFSKY, Jay ; 9321 Corsica Drive, Bethesda, MD 20814 (US). TAKESHITA, Toshiyuki ; 263 Congressional Lane, Rockville, MD 20852 (US). SHIRAI, Mutsunori ; 381 Congressional Lane, Rockville, MD 2052 (US). PENDLETON, C., David ; 9100 Old Georgetown Road, Bethesda, MD 20814 (US). MARGULIES, David, H. ; 7805 Radnor Road, Bethesda, MD 20817 (US). KOZLOWSKI, Steven ; 2501 Q Street, N.W., #105, Washington, DC 20007 (US).	<b>(74) Agents:</b> SVENSSON, Leonard, R. et al.; Birch, Stewart, Kolasch & Birch, P.O. Box 747, Falls Church, VA 22046 (US).  <b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 17 March 1994 (17.03.94)	

**(54) Title:** PEPTIDES STIMULATING CYTOTOXIC LYMPHOCYTES RESPONSE TO HIV-1 GP 160

IN18-230 8/14/91 p18 PEPTIDE TRUNCATIONS PRESENTED TO PLATE BOUND H-2Dd IN BSA. READOUT BY GROWTH INHIBITION B4.2.3



**(57) Abstract**

Peptides having high activity in the eliciting of a cytotoxic T lymphocyte response to the HIV-1 envelope glycoprotein gp160 are described. The activation of 12-15 residue peptides by proteolytic degradation to shorter peptides is shown as are general techniques for characterizing such activation processes.

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/01925

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/49 C07K13/00 C12N15/86 A61K39/21 //(A61K39/21,  
31:40) C07K15/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification-system followed by classification symbols)

IPC 5 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	JOURNAL OF EXPERIMENTAL MEDICINE vol. 175, no. 6, June 1992 pages 1417 - 1422 KOZLOWSKI, S. ET AL. 'Serum angiotensin-1 converting enzyme activity processes a Human Immunodeficiency Virus 1 gp160 peptide for presentation by major Histocompatibility Complex Class I molecules' see the whole document ---	1-4, 12-41
X	WO,A,89 07112 (THE UNITED STATES OF AMERICA, DEPARTMENT OF COMMERCE) 10 August 1989 cited in the application see the whole document --- -/--	1,2,11, 12,15,16



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

14 January 1994

Date of mailing of the international search report

17 -02- 1994

Name and mailing address of the ISA

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Authorized officer

CHAMBONNET, F

## INTERNATIONAL SEARCH REPORT

International Application No

CT/US 93/01925

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	US-Published-Patent-Application-7-760530 (Berzofsky, J.A., Takahashi, H. & Germain, R.N.) (Published in NTIS GAZETTE December 15th 1992) see the whole document	1,2,11, 12,15,16
E	& WO,A,93 05812 (GOVERNMENT OF THE USA, DEPARTMENT OF HEALTH AND HUMAN SERVICES) 1 April 1993 cited in the application see the whole document	1,2,11, 12,15,16
X	--- VACCINES 91 vol. 0, no. 0 , 1991 , COLD SPRING HARBOR LABORATORY pages 1 - 7 TAKAHASHI, H. ET AL. 'A unique subunit immunogen iscom-gp160 can elicit MHC class-I-restricted hiv envelope-specific CD8-positive CTLs' see the whole document	1,2,12, 13,15,16
O,X	& MODERN APPROACH TO NEW VACCINES INCLUDING PREVENTION OF AIDS, 8TH ANNUAL MEETING, COLD SPRING HARBOR, NEW YORK, USA, SEPTEMBER 1990	1,2,12, 13,15,16
X	--- AIDS RESEARCH AND HUMAN RETROVIRUSES vol. 7, no. 2 , February 1991 page 144 BERZOFSKY, J.A. ET AL. 'Induction of cytotoxic T cells to HIV proteins' see abstract	1,2,11, 12,15,16
O,X	& ANNUAL MEETING SPONSORED BY THE LABORATORY OF TUMOR CELL BIOLOGY, NATIONAL CANCER INSTITUTE, HYATT REGENCY HOTEL, BETHESDA, MARYLAND, USA, AUGUST 11-17 1990	1,2,11, 12,15,16
X	--- JOURNAL OF EXPERIMENTAL MEDICINE vol. 171, no. 2 , 1 February 1990 pages 571 - 576 TAKAHASHI, H. ET AL. 'An immunodominant class I-restricted cytotoxic T lymphocyte determinant of Human Immunodeficiency Virus type 1 induces CD4 class II-restricted help for itself' cited in the application see the whole document	1,2,11, 12,15,16
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## INTERNATIONAL SEARCH REPORT

n International Application No

PCT/US 93/01925

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF CELLULAR BIOCHEMISTRY vol. 0, no. 16D , 5 March 1992 page 66 KOZLOWSKI, S. ET AL. 'A component of fetal calf serum processes a class I-restricted peptide into its active form' see Abstract O 430 ---	18
A	WO,A,91 02544 (INSTITUT PASTEUR, UNIVERSITE PIERRE ET MARIE CURIE) 7 March 1991 see the whole document ---	1
A	WO,A,91 14449 (INSTITUT PASTEUR) 3 October 1991 see the whole document ---	1
A	EP,A,0 402 088 (MERCK & CO. INC.) 12 December 1990 see the whole document ---	1
P,A	EP,A,0 490 667 (NIPPON MINING COMPANY) 17 June 1992 see the whole document ---	18-23
P,A	EP,A,0 518 672 (MERCK & CO. INC.) 16 December 1992 see page 2, line 22 - line 32 -----	21-23

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/01925

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark : Although claims 15-26, 28-41 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- claims 1-26, 28-41 all complete and 27 partially
- claim 27 (partially)

See PCT/ISA/206 mailed on 08/11/93

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/JP93/01925

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